Zinc finger on the pulse

Developments and implications of zinc finger technology

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Netherlands Commission on Genetic Modification (COGEM)

COGEM has the duty to advise the government on the risks of genetically modified organisms and to report on the ethical and social aspects of genetic modification (Environmental Management Act §2.3).

Cover illustration: Thomas Splettstoesser

Summary

Since the 1970s genetic modification techniques have made it possible to alter hereditary material in a desired way. Nevertheless, inducing alterations at the right place in the genome still remains a challenge. Frequently alterations are induced at undesirable locations in the genome. Emerging new techniques can overcome this drawback. An example of such techniques is zinc finger technology. A zinc finger is a DNA-binding protein that recognises specific base sequences in the DNA and binds onto them. When the first zinc finger was discovered no-one could have guessed that twenty years later many people would view the use of zinc fingers as a possible breakthrough in the field of genetic modification. Scientists now expect that these proteins will make it possible to modify hereditary material with great accuracy and efficiency.

Both the medical and plant biotechnology sectors are putting considerable effort into developing the technology. Scientists are attempting to improve the technique and the first practical applications are in sight. With this report COGEM wants to inform government of the latest developments in this field and their possible policy implications.

It is not clear whether all the uses to which zinc fingers are put will fall, or should fall, under the legislation governing genetically modified organisms (GMOs). There appear to be similarities with techniques that are exempt from the regulations. As this could lead to questions from practitioners, this report also examines the relevant legislation.

When zinc fingers are used to alter hereditary material they are coupled to an ‘effector protein’. Many of the effector proteins in use are nucleases or transcription factors. A complex consisting of a zinc finger and a nuclease is called a ‘zinc finger nuclease’ and a complex consisting of a zinc finger and a transcription factor is called a ‘zinc finger transcription factor’. When such a complex binds to a genome, the hereditary material is altered on or near the binding site (zinc finger nuclease) or the expression of the genes is influenced without the DNA being altered (zinc finger transcription factor). This makes zinc fingers important for various purposes, such as repairing DNA, replacing genes, and stimulating or inhibiting the expression of specific genes.

Researchers use zinc fingers in a wide range of applications. The medical sector is looking into the use of zinc finger genes as gene therapy agents. Attempts are also being made to develop a therapy for Aids using zinc fingers. One of the potential uses of zinc fingers under investigation in plant biotechnology is to inhibit virus infections. Scientists also want to use them to improve plant characteristics, for example by developing plants with improved nutritional value.

The technique will have to be further improved before zinc fingers can be used in a wide range of applications. At the moment zinc fingers are not specific enough to recognise just a single site in the hereditary material. If a zinc finger brings about an alteration in the genome at a non-specific site, this may cause damage to the cell.

Because the technology is at a relatively early stage of development, little is currently known about the potential environmental risks of zinc fingers. In COGEM's opinion the risks are comparable with those of other modification techniques which can also lead to unintended changes in the genome of an organism. It is possible that the risks of using zinc fingers will be smaller than the use of other techniques because zinc fingers are capable of altering DNA sequences more accurately.

The question is whether all applications of zinc fingers fall under the GMO regulations. Four different situations are described to help answer this question. These four situations were derived from the mechanism of action (the effector protein) and the form in which the zinc fingers are introduced into the host cell. The four situations are summarised in the table below.

Mechanism of action	Zinc finger nuclease		Zinc finger transcription factor	
Delivery form	Protein	Nucleic acid	Protein	Nucleic acid
Effect	Alteration of sequence	Alteration of sequence	Regulation of gene expression	Regulation of gene expression
Remark	Method displays similarities with exempt techniques			No sequence alteration, but is a GMO because a nucleic acid is added
GMO under the legislation?	Probably	Yes	No	Yes

From the table we can conclude that three of the four situations are in all probability easy to classify. However, one situation may require further investigation: the use of zinc finger nucleases introduced into the cell as a protein. As this involves changes in the hereditary material it would appear to be a form of genetic modification. However, no nucleic acid is introduced into the cell and, from a scientific point of view, the activities display similarities with chemical mutagenesis, a technique that was exempted from the GMO regulations many years ago. A significant difference is that zinc fingers are highly specific in recognising DNA sequences and altering targeted sites in the hereditary material, whereas when using chemical mutagens it is not possible to predict where the mutations will occur. The question, therefore, is whether the use of zinc finger nucleases in the form of proteins should or should not be covered by the legislation.

Furthermore, will or should activities which lead to epigenetic changes also fall under the legislation? In epigenetics the coding sequence of the hereditary material is not changed, but the intention is that the modification is passed on to the progeny.

Zinc finger technology is one of the new techniques that blur the distinction between genetic modification and conventional techniques. These techniques are testing the

limits of the legislation. To inform politicians and policy makers about this regulatory issue, COGEM is monitoring new techniques. As part of this process, in 2006 COGEM published a report on the blurring of these distinctions by new techniques in plant biotechnology. In response to this a European working group was established to investigate whether a number of techniques, including zinc fingers, should fall under the GMO legislation. This report aims to support the Dutch government and the European working group in formulating a position by highlighting the differences and similarities between zinc fingers and other new modification techniques.

Contents

1 Introduction

Since the 1970s genetic modification techniques have made it possible to alter specific sites in the hereditary material. Nevertheless, inducing the desired alterations at the right place in the genome still remains a challenge.[12] Frequently alterations are induced at undesirable locations in the genome. New techniques can overcome this drawback. An example of these is zinc finger technology.

Zinc fingers, a certain type of protein, were first discovered in the African clawed toad (*Xenopus laevis*) in 1985.[3] When the first zinc finger was discovered no-one could have guessed that twenty years later many people would view the use of zinc fingers as a possible breakthrough in the field of genetic modification. Scientists now expect that these proteins will make it possible to modify hereditary material with great accuracy and efficiency.

Zinc finger technology is promising and may be highly significant for both the medical and plant biotechnology sectors. Now that the first applications are in sight, COGEM wants to bring government up to date on this interesting innovation. This report therefore examines how zinc fingers work, their potential applications and possible pointers for improving the technique.

The development of new technologies can prompt practitioners to raise questions about the scope of the regulations governing genetically modified organisms (GMOs). The legislation could come under strain. COGEM is monitoring progress with new techniques in order to keep politicians and policy makers informed about developments. In 2006, as part of this process, COGEM published a report on new techniques in plant biotechnology.[4] This report led to the establishment of a European working group whose tasks include investigating whether a number of techniques should fall under the GMO legislation. This working group is also looking into zinc finger technology.

It is doubtful whether all cases of activities involving the use of zinc fingers will fall under the GMO regulations. COGEM therefore also examine this issue in this report. COGEM will also consider whether relaxation of the regulations governing zinc finger technology is a potential option. With this report COGEM wants to support the Dutch government in formulating a position on this issue and make a contribution to the work of the European working group.

2 Zinc fingers appear to offer a solution to technical problems

Zinc fingers recognise specific DNA sequences and are therefore useful for altering hereditary material, such as repairing errors, at the desired sites. Other possibilities include the targeted insertion of DNA fragments into the genome. Besides altering the hereditary material, it is also possible to regulate gene expression. All these properties suggest that zinc fingers provide a solution to the problems of specificity and efficiency encountered in conventional genetic modification techniques. But how do zinc fingers actually work?

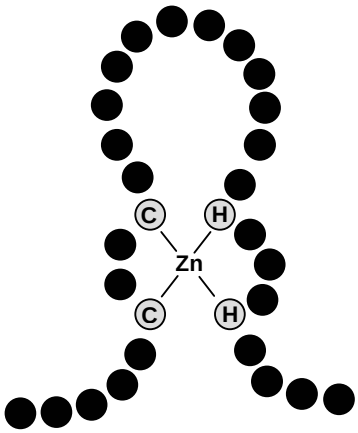

Figure 1: Diagrammatic representation of a zinc finger

2.1 Zinc fingers recognise fragments in the hereditary material

Cys_2His_2 zinc fingers, or simply ‘zinc fingers’, are a natural component of some transcription factors (factors that are involved in the process that translates information in the DNA into proteins) in most eukaryotes. Recently their presence has also been demonstrated in prokaryotes.[5,6] A zinc finger consists of 30 amino acids stabilised by a zinc ion. The three-dimensional structure of the protein is reminiscent of a finger, which is why the name ‘zinc finger’ was coined (see Figure 1).

A zinc finger is a DNA-binding domain that recognises a specific sequence of three (or even four) base pairs in the hereditary material.[5,7] Besides binding DNA, some zinc fingers are able to bind RNA or proteins.[6]

Further research has shown that linking together several zinc fingers (to produce zinc finger proteins), each with a specific binding site, creates zinc fingers that can recognise longer sequences very precisely. This greatly increases the chances of recognising a unique sequence in the genome. At the moment scientists mainly use zinc finger arrays consisting of three or four linked zinc fingers, making it possible to recognise fragments consisting of nine and twelve base pairs. Arrays consisting of six

or seven zinc fingers should be sufficient to recognise statistically unique sequences of eighteen or twenty-four base pairs in the human genome.[7,8,9] It is also clear which zinc finger can be used to bind to a desired DNA triplet.[5] This can now be used to design zinc fingers for each chosen DNA fragment.

Owing to this specific recognition of fragments in the hereditary material, extended research is currently being conducted within various scientific domains, such as the medical sector and plant biotechnology, to identify the possibilities for using zinc fingers to make changes in the genome.

We note here that in the interests of clarity, we do not refer to zinc finger proteins in this report, but simply to 'zinc fingers' for short. The term 'zinc finger' therefore covers both individual zinc fingers as well as zinc finger arrays.

2.2 Zinc fingers alter and regulate sequences in the hereditary material specifically

Simply recognising a DNA sequence is not in itself enough to alter the gene expression or the sequence of a gene. To do this a zinc finger has to be coupled to an effector protein. Depending on the desired effect, zinc fingers can be coupled to nucleases, transcription activators, transcription repressors or methylases.

2.2.1 Zinc finger nucleases cut DNA at specific sites

The effector protein being widely studied at the moment is the Fok1 endonuclease. This is an enzyme that can cleave DNA, but only when a dimer is biologically active. Coupling this nuclease to a zinc finger creates a zinc finger nuclease. Two zinc finger nucleases together are capable of inducing a double-strand break at a desired site in the DNA (see Figure 2). Such proteins can be used, for example, for the targeted repair of specific errors in the hereditary material. Given that a break can be lethal for a cell, its natural DNA repair mechanism is then triggered to repair the break.[2] Cells have two mechanisms at their disposal to do this: homologous recombination and non-homologous end-joining (see box).

One of these mechanisms is preferred, depending on the organism and the cell type.[10] In non-homologous end-joining the chromosome fragments are 'welded' onto each other, which can cause small insertions and deletions. In homologous recombination the break is repaired using a homologous template.

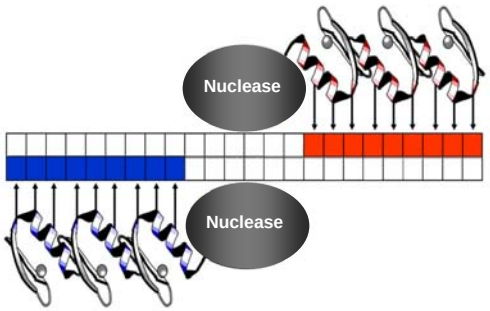

Figure 2: Diagrammatic representation of two zinc finger nucleases bound to the DNA; each zinc finger nuclease consists of three zinc fingers that together recognise nine base pairs (1). A break is induced at the site of the nucleases, which triggers the repair mechanism.

Research has shown that inducing a double strand break with zinc finger nucleases improves the otherwise highly inefficient process of homologous recombination by as much as 50,000 times.[11] This discovery has made the use of zinc finger nucleases very popular indeed.

Homologous recombination can thus be used to repair small errors in the hereditary material. In this process a 'normal' or 'healthy' DNA fragment is used as a template. This sequence may already be present in the genome, but can also be inserted as a DNA fragment together with the zinc finger nucleases.[11]

Besides repairing mutations, zinc finger nucleases are also used for the targeted insertion of a gene or DNA fragment at a specific site (also called 'gene targeting'). This technique can be used, among other things, to repair or replace aberrant genes. First a break is induced and then the introduced hereditary material is inserted at the site of the break by homologous recombination repair mechanism.[11] Activation of the repair mechanism by zinc finger nucleases stimulates the insertion of a DNA fragment by 100 to 10,000 orders of magnitude compared with a targeted insertion without the use of zinc finger nucleases.[9]

Homologous recombination
Homologous recombination is known to be a very precise mechanism for repairing breaks in DNA.[5] It is a sort of copy/paste process in which a undamaged homologous DNA sequence serves as an information source for the repair.[5] A similar sequence in the genome can serve as homologous DNA, for example the sequence on the sister chromatid.[5] Homologous recombination is by nature a very efficient process in mammal and plant genomes.[11]

Non-homologous end-joining
Another way in which plants in particular repair 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 therefore often results in deletions or insertions at the site of the break.[10,51]

Conversely, use is made of the non-homologous end-joining repair mechanism to initiate desired mutations at a specific site in the hereditary material (targeted mutagenesis).[12] The mutation then occurs at the site of the break[12] and prevents the gene from functioning properly. This allows scientists to study the function of the disabled gene.[13]

2.2.2 Zinc finger transcription factors regulate gene expression specifically

In addition to using zinc finger nucleases to induce genetic transformations in the genome, it is also possible to use zinc fingers to regulate gene expression without having to modify the gene itself.[14] In this process zinc fingers are coupled to a transcription factor.

Zinc finger transcription factors can be used in different ways to regulate gene expression. Depending on the nature of the effector protein, the binding of zinc finger transcription factors to a specific DNA sequence can either inhibit or stimulate gene expression.[14] These effector proteins therefore act as transcription repressors or transcription activators.

When using these processes, scientists are not only interested in temporarily inhibiting or stimulating gene activity, but also in studying the induction of inheritable effects. These effects are called epigenetic effects. The base sequence of the hereditary material is not altered, but the activity of the DNA is changed.[15]

2.3 Zinc fingers can be introduced into an organism in various ways

There are two ways to introduce zinc fingers and their effector proteins into a cell. The first method, which is still under development, is to introduce zinc fingers in the form of a recombinant protein. It is expected that the use of these techniques will increase rapidly.[9]

Second, the gene that codes for the zinc finger effector protein can be introduced into the cell. Although it is possible to inject the nucleic acid directly into the cell, this is often done with the use of a vector.[16,17,9] The most suitable vector depends, among other things, on the type of cell and the organism receiving the zinc finger. Roughly speaking, a distinction can be made between plant cells and the cells of mammals, other animals and microorganisms. The most commonly used vectors for the second group are plasmids and attenuated viruses. Zinc fingers and their effector proteins can be introduced into plant cells in various ways. For example, scientists use the bacterium *Agrobacterium tumefaciens* or the ‘particle bombardment’ method. It is also possible to introduce only plasmid DNA into a cell, after which a plant can be cultured from the resulting transformed cell. It is also possible to alter just a few cells of a plant, for example some leaf cells.

3 Promising applications of zinc fingers

Over the years the field of genetic modification has developed rapidly and the technique has been further refined. Scientists are always looking for quicker methods and more targeted modification techniques. The ultimate aim is to improve the technique to such an extent that the right alterations can be made very efficiently and at any desired site in the genome. The use of zinc fingers may represent a big step in this direction.

Currently much research is being done to improve zinc finger technology. While many experiments are on cell lines, organisms such as worms,[18] zebrafish,[16,17] fruit flies[19] and plants are also increasingly used.[20,37,38] In several of the organisms it has proved possible to alter the hereditary material by directly injecting zinc fingers into the embryo.[16,17,19] The resulting alterations to the DNA are then passed on to subsequent generations. Zinc finger technology has now been taken up in the medical and plant breeding sectors, where it may eventually open up promising new applications.

3.1 Zinc fingers hold promise for applications in the medical sector

Much research is being done on the use of zinc fingers in the medical field. In most cases, though, the research is in the early stages of development. Nevertheless, several studies have already been described in which the use of zinc finger nucleases and zinc finger transcription factors is far advanced and in which they have been used in human cells.[21,13,22] A few applications of zinc finger nucleases and zinc finger transcription factors are described below.

3.1.1 Use of zinc finger nucleases as gene therapy agents

In every medical therapy the aim is to obtain the best possible effect at the lowest possible risk to the patient. In gene therapy, for example, hereditary material is introduced into cells in order to insert a ‘healthy’ gene or repair a faulty gene. In the clinical studies now taking place the hereditary material is usually altered using certain attenuated viruses. The genome of the virus, which contains the desired gene, is inserted into the human genome. The therapeutic gene thus remains active and stable in the cell.

A risk of this strategy is that the gnome of the attenuated virus will be inserted in a wrong place in the genome. This may activate oncogenes, which in turn may cause the treated cell to divide and grow into a tumour. This has proved to be a real risk in practice. In a gene therapy study in France and the United Kingdom, five of the twenty patients with X-SCID (X-Severe Combined Immunodeficiency Syndrome) contracted leukaemia after treatment.[23] The introduced gene had been inserted near to an oncogene involved in the synthesis of blood cells. It is highly likely that these patients contracted leukaemia as a result of over-activation of this gene. Partly because of such adverse consequences, the high expectations for gene therapy have been tempered somewhat in recent years. However, given the specificity of zinc finger nucleases and the relatively high efficiency of gene correction, zinc finger nucleases should make a positive

contribution to the development of gene therapy. Zinc finger technology therefore appears to be a promising alternative to current gene therapy strategies.[24]

A first step in this direction was made in 2005 with the demonstration of efficient gene correction in human cells by zinc finger nucleases.[1] In a preclinical study the mutation in the defective *IL2Rγ* gene, responsible for the X-SCID disease, was repaired in about 18% of the cells treated with zinc finger nucleases.[1] The zinc finger nucleases were introduced into the cells via plasmids, where the error in the genome was repaired.[1] A few years later this experiment was repeated with a viral vector instead of a plasmid and the mutation was repaired in 39% of the cells.[25]

Furthermore, the first steps have been taken in developing a potential therapy against the virus that causes AIDS (HIV-1). The aim is to make certain cells resistant to infection with HIV-1 by altering a cell receptor in such a way that HIV can no longer bind to the cell. This approach is based on the fact that individuals with a naturally altered cell receptor appear to be immune to HIV infections.[26] When zinc finger nucleases are used to make such alterations in cell lines, these have been found to be insensitive to HIV-1 over a period of at least 70 days.[26] The therapy is most effective when zinc finger nucleases are introduced into the cell using an adenovirus, a virus that does not integrate into the genome.[26] The biotechnology company carrying out this research, Sangamo Biosciences, has recently obtained permission in the United States to conduct clinical gene therapy trials to further investigate this. Sangamo has many patents for the development and use of zinc fingers.

3.1.2 Gene regulation by zinc finger transcription factors as therapy

Zinc finger transcription factors are being increasingly used to regulate genes, both in laboratory experiments and in clinical trials. The use of zinc finger transcription factors is considered to be highly valuable because many diseases are the result of inactivated genes or genes with aberrant expression.[14,28] The mechanism of action of zinc finger transcription factors was first demonstrated in 1994 in a mouse tumour cell line in which expression of the oncogenes could be specifically blocked. This prevented any further growth of the tumour cell line.[27] Moreover, zinc finger transcription factors are also being used to develop a therapy against AIDS. Suppression of the HIV-1 promoter by zinc finger transcription factors makes it possible to strongly inhibit viral replication in the cell lines.[28]

Meanwhile, in the United States the first clinical trial involving the use of zinc finger transcription factors have been registered with the Office of Biotechnology Activities of the National Institutes of Health.[29] One of these studies is a phase I clinical trial for patients with ischaemia.[30] These patients have damaged blood vessels, which restricts the flow of blood to the limbs. There is no adequate treatment for this disorder and in the most severe cases the disease can progress to an extent that makes it necessary to amputate the affected limbs. The clinical study is attempting to limit the effects of ischaemia by stimulating the formation of new blood vessels (angiogenesis). This study was launched following positive results from research on mice. Using zinc finger

transcription factors scientists were able to stimulate a gene (coding for Vascular Endothelial Growth Factor) that is important for angiogenesis.[31]

Phase II clinical trials have also been started on the regulation of the same protein in the treatment of diabetic neuropathy (disorder of the sensory nerves), a complication in diabetes patients.[32] Interim results show that the treatment is safe, but no differences could be detected between patients treated with zinc finger transcription factors and patients treated with a placebo.

3.2 Zinc fingers hold promise for applications in plant biotechnology

The plant breeding community is constantly striving to improve existing crop varieties. With the advent of genetic modification the technique was used to improve crops and develop varieties with new characteristics. However, with the current modification techniques it is difficult to predict where the desired gene will be inserted into the plant genome. This is a disadvantage because plant genomes have regions with high and low transcriptional activity, which means that the integration site has an effect on the expression of the desired gene (the 'position effect'). For breeders it would be an advantage to be able to accurately determine the insertion site and thus allow them to predict the degree of expression with greater accuracy.

In addition, targeted insertion can prevent the insertion of a gene into a coding region of the genome, which can lead to inactivation of genes or the formation of new gene combinations. In other words, if the gene insertion site can be determined, the characteristics of the plant can be predicted more accurately and the development process of the plant will be quicker and therefore more cost efficient. The process can even be shortened by one to two years.[33]

In by far the majority of studies zinc fingers are coupled to nucleases or transcription factors. However, it is also possible to bind zinc fingers to a fluorescent label.[34] Live imaging techniques can then be used to reveal a specific sequence in the genome, for example of a plant, allowing a sequence to be examined microscopically.

The great potential of these developments is illustrated by the fact that the biotechnology company Dow AgroSciences started using zinc finger technology a few years ago,[35] under an agreement with Sangamo BioSciences. In 2008 Sangamo announced that zinc finger technology could be used, among other things, for the rapid and precise development of new or improved foodstuffs.[33] Both companies have announced that they intend to develop products that are healthier or have a higher nutritional value. They also want to use the technology to make plants that are resistant to a range of insect pests and diseases and have plans to develop plants 'to order' for use in the biofuels industry.[33]

3.2.1 Improving zinc finger nucleases and identifying gene function

In the last twenty years many attempts have been made to achieve targeted insertion in plants.[10] Initially the technique was very inefficient, which made it unlikely that it would find any practical applications. The main reason was that plants prefer to integrate DNA into the genome via non-homologous recombination. After considerable further research the literature suggests that the efficiency of insertion is dramatically

increased through the use of zinc finger nucleases.[36] For example, zinc finger nucleases have been successfully used to achieve efficient insertion of genes that code for herbicide tolerance at the desired site in the maize genome.[37] The progeny of these plants also express the relevant characteristic.[37]

In plant biology zinc finger nucleases are also used to induce specific mutations. One of the reasons scientists use this method is to change gene functions. An example is inducing point mutations in a few genes of the tobacco plant to make it tolerant to certain herbicides.[38]

Scientists are also trying to induce specific mutations in order to discover the functions of specific genes. One method for analysing gene function is to create loss-of-function mutations to determine the function of the gene in question. These mutants are created by targeted mutagenesis using zinc finger nucleases and the non-homologous end-joining repair mechanism, which causes the mutation to occur at the site of the break.[10] The mutation then prevents the gene from functioning optimally.

3.2.2 Zinc finger transcription factors improve plant characteristics

Besides zinc finger nucleases, zinc finger transcription factors are also used in plant biotechnology. Researchers are using zinc finger transcription factors to influence gene expression and thus obtain a plant with the desired phenotype.[39,40] This is facilitated if effective zinc finger transcription factors are available, which is not always the case. Researchers are therefore attempting, by trial and error, to find effective zinc finger transcription factors. Large numbers of plants are each given a specific zinc finger transcription factor and then studied to identify which plant exhibits the desired phenotype. The effective zinc finger in this plant can then be isolated for use in further research.[41]

Although current research focuses primarily on improving the technology for use in plants, a few applications have already been described. Earlier in this chapter we discussed the inhibition of viruses in humans; the first steps in this direction have now also been made in plants. An example is the use of zinc finger transcription factors to inhibit an infection with beet severe curly top virus. Zinc finger transcription factors have been shown to inhibit the replication of the virus in the model plant thale cress (*Arabidopsis thaliana*). This gave the plants resistance against the virus and 84% of them showed no observable symptoms.[42]

There is also much conjecture about using zinc finger transcription factors to obtain plants with improved flavour or nutritional value, as well as to suppress the expression of genes coding for allergenic proteins. Moreover, it is thought that zinc finger transcription factors could be used to stimulate or inhibit the expression of genes involved in various disease and stress tolerances.

4 Off-target effects and environmental risks of zinc fingers require attention

Judging by the literature the expectations for zinc fingers are high.[43] At the moment, though, the technology has disadvantages and limitations. The use of zinc fingers requires considerable knowledge about zinc finger biology and it is difficult to design suitable nucleases that can induce specific breaks in the DNA. Moreover, it is harder to engineer transcription factors that contain specific zinc fingers for plant systems than for mammal systems.[40] Further research will be needed before the techniques can be used in a wide range of applications. Moreover, experience with the use of zinc fingers is limited, which makes it difficult to assess the potential risks.

4.1 Use of zinc fingers may involve off-target effects in the organism

Current knowledge indicates that the main disadvantages of zinc fingers are related to off-target effects. These occur when zinc fingers bind non-specifically to hereditary material, which can result in undesirable alterations.[5,9,13] Off-target effects may be caused in a number of ways. A few of these are explained below.

One of the most important causes of off-target effects of zinc fingers is their lack of specificity. The specificity of zinc fingers must be high enough for the hereditary material to be recognised at just one site. If the specificity is not high enough, the zinc finger can bind to a DNA fragment that is not entirely homologous to the target segment. This binding alone can be enough to disrupt normal gene expression or cellular processes.[13] In addition, inducing a break at an undesirable site in the genome can also have damaging consequences for the cell.[5,9,13]

At the moment the specificity of zinc fingers is not always high enough, which results in a zinc finger nuclease not only inducing a break in the genome at the desired site, but also cleaving the DNA elsewhere in the genome. These breaks are mainly repaired by the imprecise non-homologous end-joining mechanism, leading to undesirable mutations as a result. Research into reducing off-target effects is continuing undiminished.[44]

Off-target effects can also arise when there are too many zinc fingers in a cell. Once zinc fingers have been introduced into a cell in the form of nucleic acid, zinc finger proteins are produced. There is a chance that large quantities of proteins will be formed, whereas just a few molecules are enough to induce an effect. Experiments with fruit flies have shown that overproduction of zinc finger proteins can lead to cell death. The high concentration causes non-specific binding of zinc fingers to the DNA, which can lead to undesirable breaks at various sites in the genome.[5] Moreover, there is a possibility that the cell will die if too many breaks are made in the hereditary material.[5]

For both gene targeting and targeted mutagenesis it is always desirable that zinc fingers are active for just a brief moment. For this reason it is possible that in future zinc fingers will be delivered more often in the form of plasmid DNA, proteins or mRNA that encode for zinc finger nucleases.[9]

4.2 Off-target effects are difficult to demonstrate

As described above, zinc fingers can cause off-target effects. It is difficult to detect these effects. In the event of genetic transformations a genome analysis may provide a definitive answer, but if non-specific binding by zinc fingers leads to epigenetic changes it is very difficult indeed to check for off-target effects.

4.3 Environmental risks of zinc finger technology resemble those of other modification techniques

When appraising conventional transformation techniques an environmental risk assessment is performed to determine whether the use of GMOs will present risks to human health and the environment. But what risks do zinc fingers pose?

Zinc finger nucleases induce mutations in a specific way. Although at the moment off-target effects cannot be ruled out completely, scientists are trying to further improve the technology. In COGEM's opinion, the risks are comparable with those of other techniques used in genetic modification. It is known that conventional techniques can also cause unintended mutations in the genome of organisms. COGEM notes that many plants are developed in conventional plant breeding using mutagenesis, which is a non-specific technique. Many crops with improved characteristics have been obtained through the use of chemical mutagens, such as ethyl methane sulphonate (EMS), X-rays or UV radiation. Examples of these characteristics are increased yields, cold tolerance and disease resistance.[35] Use of irradiation methods can lead to major changes in the genome, such as deletions, and the use of EMS frequently causes point mutations. Genetic modification techniques can also be used to introduce or remove genes. Genes are inserted into the genome at random sites, which can lead to unintended effects.[45] It is widely expected that in the future the insertion of genes using zinc fingers will be more site-specific than with current genetic modification techniques. In all probability the risks will then also be smaller.

A further question is whether risks to people will arise during the use of zinc fingers in plants as well as humans. A possible case where such risks could arise is the use of zinc fingers in gene therapy if nursing staff are contaminated following an accident with a hypodermic needle. If the zinc finger is in the form of a protein, the consequences are not likely to be any different from those of other types of therapy. Such incidents are also comparable with accidents that might arise from the use of restriction enzymes (enzymes that can each break the hereditary material). These enzymes have been used for years in many laboratories around the world without any reports of adverse consequences. If the zinc finger is in the form of DNA, risks cannot be ruled out entirely. The DNA could possibly insert itself into the genome.

Finally, a zinc finger introduced into a patient may subsequently be 'shed' (secreted). It is no known whether this would present a risk to the patient's environment or the wider environment.

5 Zinc fingers may affect the GMO regulations

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'.[46] Moreover, genetic modification is also involved in the use of recombinant DNA and RNA techniques in which use is made 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 experience had been gained with these techniques over many years. Some of these techniques have been in commercial use for plant breeding since about 1930.[47] Exempt techniques are cell fusion and protoplast fusion between crossable relatives, self-cloning of non-pathogenic microorganisms, and the use of mutagens and chemical agents.

The question is whether all applications of zinc finger technology fall under the definition of genetic modification, and therefore under the GMO legislation. This question does not always have a simple or clear-cut answer, partly because both legal and scientific arguments are involved.

5.1 Do zinc fingers fall under the GMO regulations?

Scientists are attempting to regulate gene expression with zinc finger transcription factors. In contrast, zinc finger nucleases are used to alter hereditary material. This may involve changing a sequence or the deletion or addition of hereditary material. Whether operations fall under the GMO regulations depends not only on the type of zinc finger involved, but also on the form in which zinc fingers are introduced into the cell. A distinction can be made between delivery in the form of a protein and delivery in the form of a nucleic acid. This gives rise to four situations, which are described below and summarised in Table 1.

5.1.1 The use of zinc fingers in nucleic acid form

Under the European legislation introducing zinc fingers into the cell as a nucleic acid is a case of genetic modification[47] because nucleic acid is introduced into a host cell, and this nucleic acid can lead to alterations in the hereditary material. This therefore applies to both zinc finger nucleases and zinc finger transcription factors, despite the fact that the latter situation involves the regulation of gene expression and not an alteration in the DNA sequence.

5.1.2 The use of zinc fingers in protein form

This section examines the question whether the use of zinc finger transcription factors in protein form and zinc finger nucleases in protein form fall under the GMO regulations.

The use of zinc finger transcription factors in protein form

If zinc finger transcription factors are introduced into a cell in protein form, there will be no alterations to the DNA sequence in the hereditary material because zinc finger transcription factors are used to regulate gene expression. There would therefore seem to be no genetic modification involved. Under this line of reasoning these activities do not fall under the EU directives.

A further consideration is that some medicines on the European market have a similar effect and do not fall under the GMO regulations. An example is Vidaza (azacitidine), which blocks the synthesis of DNA and RNA. It is used among others in the treatment of certain forms of cancer.[48,49] These medicines are another indication that the boundaries between conventional techniques and genetic modification are becoming blurred.

The use of zinc finger nucleases in protein form

The use of zinc finger nucleases in protein form would appear to involve genetic modification, given that it involves alterations in the hereditary material. The minimum effect of zinc finger nucleases is the induction of a break in the genome, which would appear to put them within the scope of the European legislation and therefore the GMO regulations.

However, it is questionable whether the use of zinc finger nucleases introduced into the cell in protein form actually does fall, or should fall, within the scope of the GMO regulations. First, no nucleic acid is introduced into the cell. Second, from a scientific point of view the effects achieved with zinc fingers are similar to those achieved using chemical mutagens, a technique which is exempt from the legislation. In relation to this it should be noted that zinc finger nucleases are used to make highly targeted alterations in the hereditary material. When chemical mutagens are used it is not possible to predict where the mutations will occur in the genome. Given that the use of zinc finger nucleases in protein form is largely comparable to the use of chemical mutagens, the question is whether activities involving the use of zinc finger nucleases in protein form should be covered by the GMO regulations, or whether this line of reasoning provides sufficient justification for exemption.

Another aspect of the use of zinc finger nucleases in protein form is that currently they are used to induce small mutations. But what if in future the technology is used to delete a whole gene? Will this still be comparable to chemical mutagenesis? Radiation methods can also be used to make large changes in the DNA. Should they fall under the legislation or not? And where is the dividing line? These questions touch upon the issue of whether the modification process or the characteristics of the modification product should be the determining factor. We explore the question in section 5.3.

Mechanism of action	Zinc finger nuclease		Zinc finger transcription factor	
Delivery form	Protein	Nucleic acid	Protein	Nucleic acid
Effect	Alteration of sequence	Alteration of sequence	Regulation of gene expression	Regulation of gene expression
Remark	Method displays similarities with exempt techniques			No sequence alteration, but is a GMO because a nucleic acid is added
GMO under the legislation?	Probably	Yes	No	Yes

Table 1: Summary of the different situations

5.2 Epigenetic changes by zinc fingers

A growing amount of research has been done in recent years into the possibilities of making epigenetic changes in the genome using zinc finger transcription factors and nucleases. Although none of this leads to a change in the DNA sequence, the intention is that the change in the cell is retained and that it will be passed on to progeny.

Having said that, in plants it is possible at a later date to delete the gene coding for the zinc finger and the effector protein, both of which have been introduced to induce the epigenetic change. To do this the modified plants are first crossed with unchanged plants. Then a selection is carried out on the progeny that possess the epigenetic change, but not the gene coding for the zinc finger or the effector protein. The result is a descendant that does not possess any transgenes, but whose parent plants were manufactured using recombinant DNA techniques.

The question is whether activities which lead to epigenetic changes will or should also fall under the GMO regulations. It is conceivable that the gene expression of a cell will be altered by the introduction of zinc finger transcription factors in protein form. In this case no nucleic acid is introduced into the cell and no changes are made to the hereditary material, but there is the possibility of a heritable effect occurring. Although the result of the epigenetic change may be the same as the result of genetic modification, it appears not to be. This is another example of the blurring of the distinction between genetic modification and conventional techniques.

In 2006 COGEM commissioned a study of the state of the art in the field of epigenetics.[50] At that time it was concluded that it was too early to make any further pronouncements on the potential environmental risks. However, they considered it necessary to continue to monitor developments in this field. COGEM plans to critically examine the subject of epigenetics again this year and review the current state of the art. The study will also review developments against the current GMO regulations.

5.3 Relaxation of the regulations governing zinc fingers may be possible

As mentioned above, many activities involving zinc fingers appear to fall under the GMO regulations. However, there are applications in which the distinction between genetic modification and conventional techniques is becoming blurred. In addition, there are applications that display similarities with techniques that are exempt from the GMO regulations. An example is the use of zinc finger nucleases in protein form because the results are comparable with the effect of chemical mutagenesis. With respect to this we note that the use of zinc fingers enables more targeted mutagenesis, which probably makes potential risks to human health and the environment smaller than the use of chemical mutagenesis. However, the use of chemical mutagenesis is exempt from the regulations in view of the many years of experience with the technique. No such extensive experience has yet been gained with zinc fingers.

A further relevant consideration regarding a possible relaxation of the regulations governing zinc fingers is the way in which the GMO regulations are currently implemented. In Europe a decision was made to design specific legislation for GMOs in order to guarantee human and environmental safety. The reasoning behind this was that genetic modification can be used to create organisms with new characteristics that have never before existed in the environment. Such legislation is known as process-based legislation because the method of production – the process – is the reason for drawing up the legislation. The United States, Canada and some other countries have chosen to include GMOs within the general legislation. In these cases the characteristics of an organism – the product – are the reason for implementing the legislation (product-based legislation), regardless of the techniques used and the way in which the organism is produced. In these definitions, therefore, ‘process’ stands for the method or technique used to manufacture an organism and ‘product’ stands for the organism and its characteristics. Some modern biotechnological techniques lead to products, such as crops, that cannot be distinguished from the products of conventional breeding; the only difference is the way they have been produced. In the EU these crops fall under the GMO regulations, but in other countries, like the United States and Canada, these products or crops are not considered to be GMOs.

Under a strict process approach many examples of zinc finger technology fall under the GMO regulations, although the end result – the product – may not be recognisable as a GMO. This applies to several new techniques. In June 2009 COGEM published a report on this issue.

As mentioned earlier, the aim is to develop zinc fingers that are able to induce changes at specifically chosen sites in the genome in a highly efficient manner. However, at the moment the specificity is seldom high enough for zinc fingers to induce changes at a unique site in the genome.[9,13] Scientists expect that zinc finger specificity will improve considerably in future; fragments of eighteen or twenty-four base pairs should be enough to recognise a statistically unique sequence in the human genome.[9,8] In addition, researchers are also working hard to improve the vectors used. They are attempting, for example, to improve the efficiency and regulate the level of expression.[9]

If it becomes possible in future to produce highly specific zinc fingers and improved vectors, the risks to human health and the environment are expected to be reduced. Should this be the case, it may in future be possible to relax the GMO regulations for some zinc finger applications.

A European working group has been established in response to the COGEM report 'New techniques in plant biotechnology'.[4] This working group is investigating whether several new techniques, including zinc fingers, should fall under the legislation on GMOs. With this report COGEM wants to help the working group to come to a position on this issue.

6 Conclusion

Zinc fingers are a new method for changing hereditary material. Most activities involving zinc fingers fall under the GMO regulations. Only the use of zinc finger transcription factors introduced into the cell in protein form seems not to involve genetic modification.

The regulations on these techniques could possibly be amended in the short or longer term. First, because a few activities could be exempted because of their similarities with other exempt techniques, such as chemical mutagenesis. Second, in the future zinc finger technology will most probably be considerably improved, further reducing the potential risks. It is possible that this could justify a relaxation of the regulations. But we are not that far down the line yet.

With this report COGEM wants to keep the government appraised of the current situation regarding zinc fingers. In addition, the report can contribute to the formulation of a national and European policy position on whether zinc fingers should fall under the GMO regulations.

COGEM will continue to monitor progress with new techniques in order to report on the possible consequences for human health and the environment, and their implications for the GMO regulations, in a timely manner.

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