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The Minister of Housing, Spatial Planning
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DATUM July 1st 2010
KENMERK CGM/100701-03
ONDERWERP Advice and report 'The status of oligonucleotides within the context of site-directed mutagenesis'

Dear Mrs Huizinga-Heringa,

On a request from the ministry of VROM as to whether an oligonucleotide is a recombinant nucleic acid, COGEM advises as follows.

Summary
COGEM has been requested by VROM to advise on the question whether an oligonucleotide is a recombinant nucleic acid. The answer to this question is important for deciding whether GMO legislation applies to plants developed with targeted mutagenesis.
'Classical' mutagenesis has been exempted from European GMO legislation. Classical mutagenesis employs chemical or radioactive mutagens to induce random deletions or reorganisations in the genome. Nowadays, it is also possible to create targeted mutations, using short nucleic acid molecules (oligonucleotides) with sequences that are near-identical to the target sequence in the receiving genome. This type of site-directed mutagenesis is generally considered safer than classical mutagenesis because it causes fewer random mutations in the genome of the plant. However, it is uncertain if exemption from GMO legislation applies to site-directed mutagenesis. This depends on whether an oligonucleotide should be considered a recombinant nucleic acid or not.
COGEM points out that the question whether an oligonucleotide is a recombinant nucleic acid cannot be answered univocally. It depends on the context in which the oligonucleotide is used, and the order of its sequence. COGEM limits the scope for its considerations to those oligonucleotides that are used to establish mutations in genome sequences in a cell. COGEM is of the opinion that an oligonucleotide used for site-directed mutagenesis should not be considered a recombinant nucleic acid.
COGEM notes that this request for advice underscores the fact that the framework and principles supporting the current European GMO legislation have been caught up by technological developments, putting the scientific basis of these regulations at stake.

Enclosed you will find the considerations made by COGEM and the resulting advice.

Yours sincerely,

Professor Bastiaan C.J. Zoeteman
Chair of COGEM

c.c. Drs. H.P. de Wijs
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The status of oligonucleotides within the context of site-directed mutagenesis

COGEM advice and report CGM/100701-03

Netherlands Commission on Genetic Modification (COGEM)

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

Translation: Play Your Word Right

Summary

COGEM has been requested by the Ministry of Housing, Spatial Planning and the Environment (VROM) to advise on the question whether an oligonucleotide is a recombinant nucleic acid. The answer to this request for advice is of importance for judging if plants developed using site-directed mutagenesis should fall under the current regulations for genetically modified organisms (GMOs). 'Classical' mutagenesis has been exempted from European GMO legislation. Classical mutagenesis employs chemical or radioactive mutagens to induce random deletions or reorganisations in the genome. Nowadays, it is also possible to create targeted mutations, using short nucleic acid molecules (oligonucleotides) with sequences that are near-identical to the target sequence in the receiving genome. Site-directed mutagenesis is generally considered safer than classical mutagenesis, because fewer random changes are made in the genome of the plant. However, it is unclear whether site-directed mutagenesis is legally exempt from GMO regulations. That depends on whether the used oligonucleotide should be considered a recombinant nucleic acid or not.

COGEM defines 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 points out that the question to determine whether an oligonucleotide should be seen as a recombinant nucleic acid does not have a straightforward answer. First, the answer depends on the context in which the oligonucleotide is used. In this advice, COGEM limits itself to a consideration of the use of oligonucleotides in site-directed mutagenesis.
Within this context, the sequence of the oligonucleotide is the central issue: does the oligonucleotide contain a combination of sequences that do not naturally coexist? Furthermore, the answer depends on the environment (the cell) in which the oligonucleotide is introduced. If an oligonucleotide that is introduced into a cell has a sequence that is identical to a part of the genome of the receiving cell, COGEM is of the opinion that this oligonucleotide is not a recombinant nucleic acid.

In oligonucleotide-directed mutagenesis, oligonucleotides are used that contain mutations compared to the known genome sequence in the cell. COGEM holds the opinion that these oligonucleotides should not be considered a recombinant nucleic acid. Individuals of the same species may also differ in genome sequences, and the oligonucleotide sequence may contain such a difference. Besides, the oligonucleotide sequence cannot be designated recombinant, because point mutations in a sequence cannot be defined as a sequence of their own.

COGEM recommends that the scope of the decision on the nature of oligonucleotides be restricted to the context of site-directed mutagenesis. If such a limitation of the scope is not possible, this could lead to unintended and undesirable effects on other types of experiments and technologies. It needs to be investigated if adjustment of the regulations or implementation of

new regulations is necessary, whether or not within the current framework of the GMO regulations.

This request underscores earlier reports by COGEM that the frameworks and assumptions of the current European GMO legislation have been outdated by technological developments. Increasingly, *ad hoc* assessments are necessary, which come with ever more elaborate descriptions of the context and conditions for an exception. This leads to an inextricable tangle of legal considerations, scientific descriptions and policy considerations that lack a scientific basis. A possible solution for this is to emphasise the risks of the final product in the legislation, rather than the risks of the process of modification.

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1 Introduction

VROM has requested COGEM to advise on the question whether an oligonucleotide is a recombinant nucleic acid. The answer to this question is important for determining the position of the Dutch government on whether plants developed using site-directed mutagenesis are covered by legislation on genetically modified organisms or not.

1.1 Request for advice

Genetically modified organisms (GMOs) are subject to European GMO legislation, described in Directive 2001/19/EC for introduction into the environment and Directive 98/81/EC for contained use of GMOs.[1,2] Some techniques and their resulting organisms are exempt from GMO legislation, including mutagenesis (see Annexes 2 and 3). For plant breeding companies, techniques that are exempt from GMO legislation are financially attractive. Placing on the market of organisms produced with these techniques does not require a GMO permit and corresponding delays and high costs. This motive also plays into the discussion on the technique of site-directed mutagenesis using oligonucleotides.

1.1.1 Mutagenesis

(Classical) mutagenesis uses a chemical mutagen or a radioisotope to make nucleotide substitutions in the DNA or to induce rearrangements in the genome. This results in numerous smaller and larger random deletions and changes, which may introduce new properties in the resulting organisms. These properties can be selected for. Mutagenesis is exempt from GMO legislation based on the general consideration that this technique has been used to develop new crops on a large scale for decades, with no apparent problems or public debate, and that omitting such an exemption would have economic consequences. At this time, about 3100 varieties of plants have been registered that are derived from mutagenesis, in over 175 species.[3]

Besides the classical form of random mutagenesis, it is now possible to make targeted mutations. This technique uses oligonucleotides with a sequence that is almost equal to the target sequence in the genome that needs to be mutated. A mutagen linked to the oligonucleotide itself induces the desired mutation. Site-directed mutagenesis is used in plants, micro-organisms, viruses and animals.

If mutagenesis uses oligonucleotides, it is not clear whether GMO legislation should be applied to this technique or not. This is because exemption from the Directives requires the following condition: that no recombinant nucleic acid may be used in the process of manufacturing the GMO. The Ministry of VROM has requested COGEM to answer the question whether an oligonucleotide should be considered a recombinant nucleic acid.

1.2 Previous advice

In 2005 and 2006, COGEM published two advices on the use of oligonucleotides. These recommendations followed the development of molecular techniques in the medical and plant

biotechnology field. The first advice charts the application of oligonucleotides and their genetic effects.[4] The second advice is a reflection on the use of oligonucleotides coupled with a mutagen for site-directed mutagenesis in plants.[5] According to COGEM, this type of site-directed mutagenesis should be considered a type of classical mutagenesis, in part because the associated mutagens (chemical molecule or radionuclide) cause the mutation and the oligonucleotide just ensures its 'localisation.'

In its advice, COGEM notes that 'classical' or random mutagenesis causes numerous random mutations in the genome by using radiation or chemical mutagens. However, oligonucleotides bind to a specific DNA sequence in the genome, allowing mutation to occur in a targeted manner. During this process, there is a minor chance that unintended modifications are introduced into the DNA, in addition to the intended mutations. Because of the increased specificity, for example by using oligonucleotides, COGEM sees fewer risks in site-directed mutagenesis than in untargeted mutagenesis. The Belgian Biosafety Advisory Council[6,7] and the Ministry of VROM[8] also hold this opinion.

The Belgian Biosafety Advisory Council advised in 2007 on *'targeted gene repair'* (site-directed mutagenesis using oligonucleotides). In its advice, the council states that 'targeted gene repair' should not fall within the scope of the European GMO legislation, because the technique is a type of mutagenesis and because synthetic oligonucleotides should not be considered recombinant nucleic acids. Additionally, it stipulates that there are scientific arguments to not let these techniques be covered by the scope of European GMO legislation.

1.3 Letter and spirit of European GMO legislation

Directive 2001/18 explicitly states that mutagenesis is excluded from the directive, provided that it does not use recombinant nucleic acids or GMOs. Strikingly, this reservation was not included in Directive 90/220[9], the predecessor of Directive 2001/18. This old directive states (in part II, Annex 1B) that mutagenesis is exempt from the directive, provided no GMOs are used as either recipient or parent organisms (see Annex 1). Apparently, there was an explicit reason to include the reservation about recombinant nucleic acids in 2001.
This raises the question whether the legislator was also thinking about oligonucleotides. COGEM points out that environmental safety considerations do not prompt this (see section 1.2). The legislator wanted to exempt mutagenesis because this technique had been used for some time, with no apparent safety hazards, and did not retroactively have to be regulated. The spirit of the law therefore seems to aim for exemption of mutagenesis.

Possibly, the legislator intended to safeguard the difference between mutagenesis and insertion of new sequence orders. It should be noted that oligonucleotides can be used to make targeted mutations, in contrast to classical mutagenesis. This means that, in theory, a successive cycle of directed mutagenesis could introduce an entirely new sequence. Here, one can think about changing the sequence of a gene or introducing the promoter in front of a genomic sequence that was previously not expressed. A possible result of this is that the distinction between the creation of point mutations and deletions, and the introduction or expression of new sequences in an organism fades. Section 5.2 discusses this further.

2 Oligonucleotides: applications and types

Oligonucleotides are short DNA or RNA molecules, composed of different nucleotides. Naturally occurring oligonucleotides include *short interfering RNAs*, *microRNAs* and *piRNAs*, ranging in length from 20 to 30 nucleotides, which regulate gene expression in the cell.
Synthetic oligonucleotides are widely used in research, for example as *'hybridisation probe'* or *'primer'* for amplification of pieces of DNA in a *'polymerase chain reaction'* (PCR) or to make modifications in specific parts of the genetic material of an organism. Oligonucleotides are used in the study of micro-organisms, plants, animals and man.

2.1 Definition of oligonucleotides

There is no fixed definition of oligonucleotides. However, a consensus can be derived from textbooks and scientific publications. This consensus finds that oligonucleotides can be synthetic or not, and consist of DNA, RNA or both types of nucleic acid simultaneously. Oligonucleotides are either single-stranded or double-stranded and may contain nucleotide analogues that have been chemically modified. In addition, for the definition of oligonucleotides, the length of the molecule is important.

'Oligos' is Greek for 'few.' The various definitions of 'oligonucleotide' do not generally specify a maximum length for an oligonucleotide, but refers to a customary length, such as 2 to 30 nucleotides. In the case of double-stranded oligonucleotides, this length is measured in base pairs. These kinds of size specifications depend on what could be synthesised at the time, and what the specific scientific field considered customary or desirable for the application. In the past, oligonucleotides were primarily used as *'primer'* for amplifying DNA in a PCR or sequencing. A common length was 20 to 25 nucleotides. Nowadays, the technology of synthesising stretches of DNA and RNA is so advanced, that nucleic acids up to 120 nucleotides in length can be manufactured easily. Larger synthetic molecules are made by ligating oligonucleotides together. For experiments inducing sequence changes in bacteria, oligonucleotides of 70 to 120 nucleotides are used, while in mammalian cells, oligonucleotides of 25 to 45 nucleotides are optimal.[10] These differences are reflected in the different definitions or designations for oligonucleotides that can be found in (semi-)scientific literature, depending on the background of the author and the state of affairs at the time.
However, to distinguish between short nucleic acids (oligonucleotides) and longer, often coding, nucleic acids, a clear standard for the length of an oligonucleotide in GMO legislation is important. COGEM recommends that for GMO legislation, the maximum length of an oligonucleotide should be defined as approximately 120 nucleotides. Based on the above, COGEM defines an oligonucleotide as follows:

> *'An oligonucleotide is a single- or double-stranded molecule consisting of different nucleotides (or their 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.'*

2.2 Applications and types of oligonucleotides

2.2.1 Mutagenesis using oligonucleotides

Oligonucleotides are commonly used in medical and plant biology research as a system to introduce modifications or mutations into the DNA of one or more cells. The modifications may be intended to create a mutation, but also to remove an existing mutation and repair gene function. Oligonucleotides are introduced into the cell that differ by one or several nucleotides from the sequence of the target DNA in the cell. The process by which these oligonucleotides can cause modifications remains largely unclear, but employs the DNA repair mechanisms of the cell. The oligonucleotide hybridises with the complementary nucleic acid, creating a mismatch. Enzymes repair this mismatch, which usually results in a restoration of the original sequence of the DNA, but in some cases may lead to a mutation, deletion or insertion in or of the DNA. The efficiency of site-directed mutagenesis varies greatly between organisms (plants, bacteria, animals, etc.) and also depends on the design and type of the oligonucleotides used.

Different types of oligonucleotides are used for mutagenesis, including chimeric DNA/RNA hybrids. These oligonucleotides are partially double-stranded nucleic acids of about 25 nucleotides long, consisting of a single-stranded RNA molecule hybridised to a complementary single-stranded DNA molecule. Examples of other types of oligonucleotides are single-stranded DNA oligonucleotides that bind specifically to the DNA double helix (*'triple helix forming oligonucleotides'* (TFO)) or single-stranded DNA oligonucleotides that are chemically modified. Chemical modification of oligonucleotides is intended to counter degradation in the cell and prevent inclusion in the genome. Two examples of chemically modified oligonucleotides are *'locked nucleic acids'* (LNAs), which have a more stable structure because of an additional methyl group, and nucleotide analogues in which an oxygen atom is replaced by a sulphur atom (the *'phosphorothioates'*).

Depending on the type of oligonucleotide that is used, often another name for site-directed mutagenesis is used. In this advice, COGEM includes all these different types under the generic terms 'oligonucleotides' and 'site-directed mutagenesis' (*'oligonucleotide-directed mutagenesis'* or *targeted mutagenesis'*).

2.2.2 Other applications of oligonucleotides

In addition to mutagenesis, oligonucleotides can be also used for other purposes. An example of a medical application is administering oligonucleotides to patients to generate an immune response. This technique uses CpG oligonucleotides and is used for cancer therapy, among other things. In addition, single-stranded DNA or RNA molecules called 'aptamers' are designed specifically to bind to proteins, nucleic acids, cofactors and chemicals. These oligonucleotides are used in diagnostics, therapy and research.

Regulation of gene expression is the third most common application of oligonucleotides. This application is modelled on the natural system of RNA interference, which prevents protein synthesis. In this technique, oligonucleotides are brought into the cell that are complementary to a particular part of a messenger (m-)RNA. After hybridisation, the target mRNA molecule is degraded. For regulation of gene expression, primarily single-stranded or double-stranded RNA oligonucleotides are used.

3 Recombinant nucleic acid: definition

Although the term 'recombinant DNA' is established among scientists and in legislation, it is difficult to give a univocal definition. Several descriptions and nuances are in circulation, partly caused by new scientific insights and interpretations. The simplest definition of recombinant DNA is a DNA molecule in which a piece of 'foreign' DNA is inserted. 'Foreign' refers to a DNA sequence that does not naturally occur in conjunction with the rest of the DNA sequence. Sometimes, it is also stated that recombinant DNA is a type of artificial DNA.
Probably the best definition is given in the authoritative text book 'Biochemistry'[11]: *"Recombinant DNA (rDNA) is a form of DNA that does not exist naturally, but is created by combining DNA sequences that would not normally occur together."* COGEM regards this definition as suitable for recombinant nucleic acid in the following form:

> *'Recombinant nucleic acid is a nucleic acid with a nucleotide sequence that does not occur naturally, but is created by combining nucleic acid sequences that do not naturally occur next to each other.'*

4 Is an oligonucleotide a recombinant nucleic acid?

The question whether an oligonucleotide is a recombinant nucleic acid, does not have a straightforward answer. Partly because no legally established scientific definitions exist, there is a strong legal perspective to the question. The following sections describe possible arguments that can be used when answering this question, and their consequences.

4.1 Semantics as guiding element

The word recombinant is a combination of *re-* (again, back) and *combine*. This implies that a recombinant nucleic acid is composed of two or more pieces of DNA or RNA. One can reason that a synthetic oligonucleotide is synthesised by linking nucleotides, and therefore no recombination of existing pieces of DNA or RNA takes place. This leads to the postulation that a synthetic oligonucleotide is not a recombinant nucleic acid.

The Belgian Biosafety Advisory Council seems to endorse this reasoning in an explanation of its article (personal communication Dr. D. Breyer), by stating that a recombinant nucleic acid should be composed of genetic material from two different sources (organisms). Because an oligonucleotide used for mutagenesis is always synthesised chemically, and not composed from various sources, the Belgian Advisory Council does not consider oligonucleotides to be recombinant nucleic acids.

However, a different reasoning might be that synthesising an oligonucleotide means recombining it out of single nucleotides. After all, is there a lower limit to the size of the pieces that are being

recombined? According to this argumentation, a synthetic oligonucleotide is by definition a recombinant nucleic acid.

Another disadvantage of a 'semantic' approach, concerns the emphasis on 'synthetic.' Because of this, the process by which the oligonucleotide is obtained takes centre stage. However, 'natural' strands of DNA and RNA can be divided into smaller fragments mechanically or enzymatically, making a synthetic oligonucleotide indistinguishable from a 'natural' oligonucleotide. This contributes to uncertainty about whether the process by which an oligonucleotide is made should be paramount, rather than the properties of the oligonucleotide. Spotlighting the process ignores the biological or intrinsic value of nucleic acids in molecular biology: the sequence order of the nucleic acid. The sequence order determines the coding properties of DNA or RNA, and thus its importance. It can be argued that recombinant refers to (re)combining different (coding or noncoding) sequence orders, and less to combining physical pieces of DNA. Indeed, recombination is carried out to combine specific sequences (or encoding properties). The physical properties of DNA molecules do not differ, but their sequences do. This links back to the previously mentioned definition in *Biochemistry*.

4.2 Sequence homology as distinctive feature

The sequence order of an oligonucleotide may be fully identical to a 'natural' sequence in a genome. In that case, there is no new combination of sequence orders that do not naturally occur next to each other. Thus, such an oligonucleotide cannot be designated recombinant.

However, this is context dependent. A piece of DNA having a sequence order that occurs in a bacterium is a 'natural' sequence. However, if this piece of DNA is introduced into the cell of a plant, many scientists will refer to it as recombinant nucleic acid, because the sequence differs from the sequences in the plant genome. The sequence of the oligonucleotide is foreign to the environment (the plant cell) in which it is introduced.
When incorporating a 'foreign' DNA sequence into a plant genome, sequences are always recombined, and a GMO arises. But in a situation where the recombinant nucleic acid is transiently present in the cell, the inserted sequence may also be regarded as recombinant.

In summary, it cannot be said that an oligonucleotide is or is not a recombinant nucleic acid by definition. This depends on the context in which the oligonucleotide is used.

4.2.1 Two different sources is not the same as two different organisms

As mentioned earlier, the Belgian Biosafety Advisory Council states that a recombinant nucleic acid must consist of sequences from two different sources (organisms). The interpretation of 'two sources' may be put to question. Even a nucleic acid that consists of two sequences from one organism that do not naturally occur together - for example the promoter of one gene combined with the coding sequence of another gene - will be seen as recombinant nucleic acid in the current scientific view.

4.3 The sequence of an oligonucleotide may differ from the natural sequence

Genomic sequences from different individuals within one species naturally differ by the numerous *'single nucleotide polymorphisms'* (SNPs), DNA *'copy number'* variations and insertions and deletions (InDels). Most of the SNPs/mutations in the genome are 'silent'; they do not lead to changes in the protein sequence they encode. Some SNPs/mutations do lead to changes in protein sequences or phenotypic characteristics of the individual. These are the genetic differences and mutations plant breeders encounter by 'chance,' and which they use for further work. Such SNPs and mutations may be deliberately obtained through oligonucleotide-directed mutagenesis.

Considering the above, the following points are important when answering the question when an oligonucleotide should be seen as a recombinant nucleic acid.
Looking at natural variation between genome sequences, one can conclude that the sequence of an oligonucleotide does not need to be fully identical to the most common (or any particular) genome sequence. Indeed, within a population individuals carry mutations in their genomic sequence, so that the sequence of one of these individuals could be identical to that of an oligonucleotide in which a point mutation or deletion is present.
The mutations a researcher or plant breeder wants to incorporate may already be present in the population. Identifying these individuals, however, is very difficult, which is why site-directed mutagenesis is used. Moreover, after identifying the desired mutation, crosses need to be carried out to create a variety with the right properties. When using targeted mutagenesis in plant breeding, this is unnecessary.
Furthermore, a point mutation in a sequence cannot be considered a recombination of different sequences. After all, one or at most a few nucleotides differ from the known 'natural sequence.' This is not enough to be regarded as a distinct sequence that can be traced to a specific source.

It can therefore be said that an oligonucleotide that is substantially similar to - and only a few nucleotides different from - a known sequence should not be considered a recombinant nucleic acid.

4.3.1 Arbitrary limit of difference between oligonucleotide sequence and target sequence

An oligonucleotide that is substantially similar to a known sequence in the target organism should not be considered a recombinant nucleic acid, according to the above. However, where are the limits for what is considered similar? The use of a limit for oligonucleotides is important to clearly delineate the difference between mutations of several nucleotides and larger modifications. The choice of such a limit, which distinguishes the situations when an oligonucleotide should be considered a recombinant nucleic acid and when it should not, will always be arbitrary. When considering the specific application of mutagenesis, a limit of one in twenty nucleotides deviating from the genomic sequence could be used.

Setting an arbitrary limit of sequence similarity to determine whether an oligonucleotide is a recombinant nucleic acid also has its drawbacks. An oligonucleotide that will be used for mutagenesis may carry additional sequences beyond the genomic sequence and the changed nucleotide(s), built in with the aim to increase mutagenesis efficiency, such as *'hairpin*

structures'. These sequences have no other function, and they are not incorporated into the genome sequence. The addition of these sequences may cause the oligonucleotide to exceed the arbitrary limit, while the final result - the mutated sequence in the organism - is identical when using a normal oligonucleotide. It is difficult to rationalise why the resulting organism should be seen as GMO in one case and not in the other.
In addition, such distinctions cannot be a basis for enforcement. The final product of an optimised oligonucleotide is indistinguishable from other types of oligonucleotide-directed mutagenesis, and it is not possible to detect which oligonucleotide was used in the production of the organism.

5 Other considerations

5.1 Request for advice goes beyond plant breeding

It is important to note that the answer to this request may affect many aspects of GMO legislation. The request concerns mutagenesis under Directive 2001/18[1], within the context of plant breeding activities. Directive 2001/18 includes introduction of all GMOs into the environment, not just genetically modified plants. In addition, EU Directive 2009/41[2] on contained use comprises the same sections (Annex II, section A). This means that an exemption of oligo-directed mutagenesis does not only concern market authorisation of mutated plants, but also concerns other organisms such as bacteria and viruses, and activities in laboratories, animal housing and greenhouses.

5.1.1 Risks for humans and environment do not exceed the natural baseline

In viruses, a single point mutation may cause a markedly increased virulence. Targeted mutagenesis can therefore lead to more virulent viruses, which raises the question whether this type of experiment should be exempt from GMO legislation. However, such virus mutants also occur in nature, and can be selected in a relatively simple manner, without GMO legislation applying. This also applies to work with bacteria and other micro-organisms.
In addition, other legislation applies to activities with pathogens, providing a safety net for this type of experiments. First, there are the Health and Safety regulations (Occupational Health and Safety Act 4.84 and EU Directive 200/54/EC)[12,13] aiming to protect workers from risks related to exposure to biological agents. Several occupational groups have drawn up guidelines, such as the guidelines and protocols of the Workingparty Infection Prevention (WIP) and the Dutch Society for Microbiology (NVvM),[14] and the Dutch Society for Medical Microbiology (NVMM).[15] For work with animal pathogens or plant pathogens, legislation regarding quarantine organisms may apply.[16,17]

5.1.2 Adaptation of legislation

As previously stated, COGEM holds the opinion that the decision whether an oligonucleotide is a recombinant nucleic acid, is inextricably bound up with the context and application of site-directed mutagenesis. If the decision also applies outside the context of site-directed mutagenesis, it will need to be examined whether adaptation of the legislation is necessary to prevent undesirable and unintended consequences. For example, if synthetic oligonucleotides are not judged to be recombinant nucleic acids and this decision applies in general, this may affect the regulation of synthetic biology. COGEM stated before that for the new scientific field of synthetic biology, no new legislation is necessary, because GMO legislation also covers synthetic biology.

However, a synthetic genome can be formed by linking oligonucleotides together and introducing this DNA molecule into a yeast or bacterial cell.[18] The cell recombines the different inserted DNA molecules into a genome. Because the synthetic genome can replace the natural genome of a bacterial cell, a 'synthetic' micro-organism is created. Such activities would not be covered by GMO legislation if oligonucleotides are not seen as recombinant nucleic acid. Additional legislation would be necessary.

Moreover, if it is decided that oligonucleotides are recombinant nucleic acids, this may also lead to unintended consequences for certain experiments. Oligonucleotides are used for many purposes (other than mutagenesis and the construction of synthetic genomes) in research. There are no reasons or justification to suddenly to apply GMO legislation to these experiments.

5.2 Limit between mutagenesis and recombination is maintained

As specified in section 1.3, theoretically, using several cycles of targeted mutations, several nucleotides in a genome can be changed. These mutations may be contiguous and open up the possibility to introduce a 'new' sequence into a genome. Alternatively, a sequence in the genome that is not expressed could be provided with a promoter and so on. These modifications are not possible using classical mutagenesis, since it is not targeted.
Due to the low efficiency of site-directed mutagenesis, however, it should be noted that this is currently only a theoretical possibility. Furthermore, with each additional cycle of site-directed mutagenesis, an oligonucleotide would have to be used that deviates more and more from the known natural genome sequence, because the oligonucleotide needs to contain the previous mutations. This means that after a limited number of cycles, the oligonucleotides used would need to be regarded as recombinant, and that the resulting organism should be considered a GMO.

5.3 Enforceability and public support

COGEM points out that organisms with natural mutations (products of classical breeding), with mutations induced by radiation or chemical mutagens, are indistinguishable from organisms produced by targeted mutagenesis. Enforcing legislation that distinguishes between these different categories is therefore extremely difficult, and even impossible for import from countries with different regulations.
Besides, it is difficult for a government to explain why one of two identical products is subject to GMO legislation and its expensive security regimen, while the other is not. Even more so because the product of site-directed mutagenesis using oligonucleotides, which would fall under the regulations, is deemed safer than the product that is exempt.

6 Report: Framework of the regulations is outdated

COGEM has previously reported that the European legislation for genetically modified organisms (GMOs) is no longer in step with the scientific insights and developments in biotechnology.[5,19] This creates confusion about what should be considered a genetically modified organism (GMO). The current request is an example of this.

In European GMO legislation, it has been decided to set up specific legislation for GMOs based on a 'process approach.' The reason or cause for regulation lies in the way the organism was made, instead of in a modified or new feature of an organism. Classical mutagenesis (through radiation or chemical mutagens) is exempt from GMO legislation, because this technique was used before the advent of genetic modification (Directive 2001/18, consideration 17). However, inducing changes in the genome through genetic modification is always regulated and subject to the GMO safety regimen, regardless of the characteristic that is inserted. With the advance of technological developments, the distinction between genetic modification and other breeding techniques becomes smaller and less clear. Artifice is needed to underpin the existing legal distinction. The current request exemplifies this. The request for advice is aimed at making a classification and a choice based on scientific terminology. However, the terms 'recombinant nucleic acid' and 'oligonucleotide' are used more or less intuitively by scientists to indicate certain categories of nucleic acids, but they are not sharply defined. What exactly is indicated by them has changed over time. In the 80s, an 'oligonucleotide' was understood to be a DNA molecule with a length of about 12 to 20 nucleotides. This was what DNA synthesisers could make. Today, that limit has been stretched to approximately 200 nucleotides, and both RNA and DNA molecules can be made. These fragments can, in turn, be linked *in vitro* to make longer fragments. For scientific purposes, an exact definition is neither relevant nor useful.

Several other countries, like the U.S., have chosen to place GMOs under existing general legislation. If an organism has a modified characteristic, that is the reason to apply regulation. Here, the product or crop is central, regardless of how it is made. This approach is referred to as a 'product approach' or 'product based'.
COGEM has previously reported that the framework of EU GMO regulations must be revised.[19] It has been noted that the current legislation puts consumer choice, the credibility of the government and the competitiveness of European industry at stake. It has been reported that the *'Cartagena protocol on Biosafety'* offers a possible solution to the dilemma between legislation and new scientific applications. The protocol offers space for a different interpretation of the EU Directive, which will be based on 'new combinations' of genetic material. By increasing the emphasis on the fact that the plants or crops need to have new properties, a connection is made with 'product based' legislation.
COGEM reports that the request for advice underscores the need to revise the legislative framework. GMO legislation must be based on clear and transparent definitions and considerations, and must be enforceable.

7 Conclusions and advice

- COGEM points out that the frameworks and assumptions of the current GMO legislation, as they are currently interpreted and used, lead to ambiguities and are ultimately unsustainable. With the advance of technical possibilities and scientific knowledge, the legal frameworks are put under pressure. Increasingly, *ad hoc* decisions need to be made, which come with ever more elaborate descriptions of situations, exceptions and scientific techniques used in the

production process. This creates a Gordian knot of legal considerations, scientific descriptions and policy considerations. The current request exemplifies this.

- However, COGEM realises that adjustment of legislation is a complex and lengthy process. Because government and industry now face the question how to deal with the products of targeted mutagenesis (*'oligo-directed mutagenesis'* or *'site-directed mutagenesis'*), the answer cannot wait. COGEM emphasises that the answer to the question whether oligonucleotides are recombinant nucleic acids is context sensitive, and that answering this question will provide a short-term solution and will be overtaken by developments.

- The question whether an oligonucleotide is a recombinant nucleic acid, does not have a simple answer. Depending on the context, an oligonucleotide may or may not be regarded as a recombinant nucleic acid. COGEM limits the scope for its considerations to those oligonucleotides that are used to establish mutations in genome sequences in a cell.

- When answering the request for advice, according to COGEM the most important consideration is whether there is a combination of sequence orders in the oligonucleotide that do not naturally occur next to each other. Furthermore, it is dependent on the environment (the cell) into which the oligonucleotide is inserted. If a sequence is identical to the sequence order in the genome (of nucleus, chloroplast or mitochondrion) of the recipient cell, this sequence is not a recombinant nucleic acid.
  This is in line with the previous advice of COGEM on mutagenesis using oligonucleotides coupled to a mutagen (chemical or radioisotope). The sequence of the oligonucleotide is identical to the genome sequence. Accordingly, this is a type of classical mutagenesis, which is exempt from GMO legislation.

- Targeted mutagenesis (without chemical or radioactive mutagens) uses oligonucleotides that contain mutations relative to the known genome sequence. COGEM points out that differences do exist between the sequences of individuals of one species. Additionally, several point mutations in a sequence cannot be seen as a sequence originating from a different source, and therefore no recombination takes place. COGEM is therefore of the opinion that the sequence of an oligonucleotide does not need to be fully identical to the known natural sequence to be regarded as non-recombinant. As an arbitrary limit at which an oligonucleotide should not be considered a recombinant nucleic acid, a difference of one in twenty oligonucleotides could be used.

- COGEM points out that the necessity of using an arbitrary limit at which an oligonucleotide does not need to be considered a recombinant nucleic acid, will raise new problems in the near future. However, this is inherent to the current framework of the EU GMO legislation, and underscores the need to rethink and reform EU GMO legislation.

- If it is not possible to limit the scope of a decision on the nature of oligonucleotides to the context of site-directed mutagenesis, this may lead to unintended and undesirable effects on other types of experiments and technologies. It needs to be investigated if adjustment of the

regulations or implementation of new regulations is necessary, whether or not within the current framework of the GMO regulations.

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Annexes

1) Annex IA and IB of the Directive 90/220/EEC regarding the deliberate introduction of genetically modified organisms into the environment (no longer in force)
2) Annex IA and IB of the Directive 2001/18/EC regarding the deliberate introduction of genetically modified organisms into the environment
3) Annex IA and IB of the Directive 2009/41/EC regarding the contained use of genetically modified micro-organisms

1) COUNCIL DIRECTIVE 90/220/EEC of 23 April 1990 on the deliberate release into the environment of genetically modified organisms (no longer in force)

ANNEX I A

TECHNIQUES REFERRED TO IN ARTICLE 2 (2)

PART 1

Techniques of genetic modification referred to in Article 2 (2) (i) are inter alia:

(1) recombinant DNA techniques using vector systems as previously covered by Council Recommendation 82/472/EEC (;);

(2) techniques involving the direct introduction into an organism of heritable material prepared outside the organism including micro-injection, macro-injection and micro-encapsulation;

(3) cell fusion (including protoplast fusion) or hybridization techniques where live cells with new combinations of heritable genetic material are formed through the fusion of two or more cells by means of methods that do not occur naturally.

PART 2

Techniques referred to in Article 2 (2) (ii) which are not considered to result in genetic modification, on condition that they do not involve the use of recombinant DNA molecules or GMOs, are:

(1) in vitro fertilization,

(2) conjugation, transduction, transformation or any other natural process,

(3) polyploidy induction.

ANNEX I B

TECHNIQUES REFERRED TO IN ARTICLE 3 Techniques of genetic modification to be excluded from this Directive, on condition that they do not involve the use of GMOs as recipient or parental organisms, are:

(1) mutagenesis,

(2) cell fusion (including protoplast fusion) of plant cells where the resulting organisms can also be produced by traditional breeding methods.

2) Directive 2001/18/EC of the European Parliament and of the Council of 12 March 2001 on the deliberate release into the environment of genetically modified organisms and repealing Council Directive 90/220/EEC

ANNEX I A

TECHNIQUES REFERRED TO IN ARTICLE 2(2)

PART 1

Techniques of genetic modification referred to in Article 2(2)(a) are inter alia:

(1) recombinant nucleic acid techniques involving the formation of new combinations of genetic material by the insertion of nucleic acid molecules produced by whatever means outside an organism, into any virus, bacterial plasmid or other vector system and their incorporation into a host organism in which they do not naturally occur but in which they are capable of continued propagation;

(2) techniques involving the direct introduction into an organism of heritable material prepared outside the organism including micro-injection, macro-injection and micro-encapsulation;

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

PART 2

Techniques referred to in Article 2(2)(b) which are not considered to result in genetic modification, on condition that they do not involve the use of recombinant nucleic acid molecules or genetically modified organisms made by techniques/methods other than those excluded by Annex I B:

(1) in vitro fertilisation,

(2) natural processes such as: conjugation, transduction, transformation,

(3) polyploidy induction.

ANNEX I B

TECHNIQUES REFERRED TO IN ARTICLE 3

Techniques/methods of genetic modification yielding organisms to be excluded from the Directive, on the condition that they do not involve the use of recombinant nucleic acid molecules or genetically modified organisms other than those produced by one or more of the techniques/methods listed below are:

(1) mutagenesis,

(2) cell fusion (including protoplast fusion) of plant cells of organisms which can exchange genetic material through traditional breeding methods.

3) Directive 2009/41/EC of the European Parliament and of the Council of 6 May 2009 on the contained use of genetically modified micro-organisms

ANNEX I

PART A

Techniques of genetic modification referred to in point (b)(i) of Article 2 are, inter alia:

1. Recombinant nucleic acid techniques involving the formation of new combinations of genetic material by the insertion of nucleic acid molecules produced by whatever means outside an organism, into any virus, bacterial plasmid or other vector system and their incorporation into a host organism in which they do not naturally occur but in which they are capable of continued propagation.

2. Techniques involving the direct introduction into a micro-organism of heritable material prepared outside the micro-organism, including micro-injection, macro-injection and micro-encapsulation.

3. Cell fusion or hybridisation techniques where live cells with new combinations of heritable genetic material are formed through the fusion of two or more cells by means of methods that do not occur naturally.

PART B

Techniques referred to in point (b)(ii) of Article 2 which are not considered to result in genetic modification, on condition that they do not involve the use of recombinant-nucleic acid molecules or GMMs made by techniques/methods other than the techniques/methods excluded by Part A of Annex II:

1. in vitro fertilisation;

2. natural processes such as: conjugation, transduction, transformation;

3. polyploidy induction.

ANNEX II

PART A

Techniques or methods of genetic modification yielding micro-organisms to be excluded from this Directive on condition that they do not involve the use of recombinant-nucleic acid molecules or GMMs other than those produced by one or more of the techniques/methods listed below:

1. Mutagenesis.

2. Cell fusion (including protoplast fusion) of prokaryotic species that exchange genetic material by known physiological processes.

3. Cell fusion (including protoplast fusion) of cells of any eukaryotic species, including production of hybridomas and plant cell fusions.

4. Self-cloning consisting in the removal of nucleic acid sequences from a cell of an organism which may or may not be followed by reinsertion of all or part of that nucleic acid (or a synthetic equivalent), with or without prior enzymic or mechanical steps, into cells of the same species or into cells of phylogenetically closely related species which can exchange genetic material by natural physiological processes where the resulting micro-organism is unlikely to cause disease to humans, animals or plants. Self-cloning may include the use of recombinant vectors with an extended history of safe use in the particular micro-organisms.