New techniques in plant biotechnology

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Commission on Genetic Modification (COGEM)
The Netherlands Commission on Genetic Modification advises the Government on the potential risks of genetically modified organisms (GMOs) and informs the Government about ethical and societal issues linked to genetic modification (Environmental Management Act, Article 2.3).

Summary

Biotechnology offers great opportunities for plant breeding. New techniques for rapidly selecting or inducing the desired characteristics are being developed. The Dutch plant breeding sector remains aloof from genetic modification in plants. The aversion of the consumer, complicated legislation and the high costs of introducing GM crops and their products do not make genetic modification an attractive alternative to conventional breeding methods. Nonetheless, with the advance of technology, the distinction between genetic modification and other plant biotechnological techniques gradually blurs. In addition, such technological developments also outgrow the GMO legislation. At times it is not clear whether the products of some techniques are subject to the prevailing GMO legislation.

Consequently, an impasse has arisen between the Dutch government and breeding companies as the developer of new techniques in plant biotechnology. Companies are only prepared to further develop some innovations when it is clear whether they are subject to the GMO legislation or not. Being bound by EU legislation, the government says it can only make this judgement when an actual application is submitted. Thus a situation is created in which both parties are waiting for each other.

Advice

This advisory report, which to some degree has a informative character, discusses six new techniques: ‘reverse breeding’, agroinoculation, grafting on genetically modified rootstock, gene silencing by DNA methylation, the use of oligonucleotides, and specific mutagenesis with homologous recombination. These techniques were chosen as they are either in the early stages of commercial application or give insight into the problem at stake. For some of the discussed techniques, the important questions are whether they can be considered genetic modification and whether their products must be characterised as GMOs. In this respect, a progressive scale can be distinguished.

The products of some techniques, such as the offspring in case of reverse breeding, do not contain any novel characteristics, added sequences, mutations or other changes. In epigenetic mutants, no sequence changes are made in the genome, though there are heritable effects. In products of grafting, transgenic sequences may be absent but transgenic proteins or other transgenic molecules or induced effects can be present. Other products, for instance those generated by the application of mutagentia coupled with oligonucleotides, do contain mutations in the genome but that production method is similar to that of organisms exempted from the legislation. Finally, some organisms are

genetically modified but by a modification technique that dismisses many of the current technical-scientific objections.

The European legislation is based on the principle that when recombinant DNA techniques are used in the production of an organism, this organism is considered a GMO with changed genetic characteristics. Therefore this organism is subject to the GMO legislation. The underlying idea here is that the process of genetic modification is inherently unsafe and associated with risks. However, with the advance of science and biotechnology, is has become possible to use recombinant DNA techniques or genetic modification in a production process, in such a way that the resulting plant or organism does not contain any added sequences or expresses other changes. An example of this are plants that are produced with the help of reverse breeding. Based on technical-scientific grounds COGEM is of the opinion that such plants should not be seen as GMOs. If current legislation implies this is not possible, COGEM recommends that they be exempt from GMO legislation.

COGEM considers further the offspring of agroinoculated plants in principle not as GMOs. However, at this moment it cannot entirely be excluded that this offspring possesses unintended transgenic sequences after agroinoculation. COGEM will conduct further research into this. Expectations are that the results of this research will be made available at the start of 2007.

As yet it is too early for a judgement on epigenetic applications and possibly related environmental risks. The stability of epigenetic changes and the underlying mechanism of heredity are unclear at this moment. Applications are not immediately expected. Furthermore, it is uncertain whether epigenetic mutants fall within the legal scope of GMO legislation.

Whether non-modified upper stem grafted on GM rootstock and their products must be subject to the GMO legislation is principally a legal and political question. However, COGEM observes that it cannot be said that there are by definition no risks to people and the environment from the upper stem (products) grafted on GM rootstock and COGEM recommends a case by case approach.

COGEM considers specific mutagenesis with oligonucleotides a form of ‘traditional’ mutagenesis. It should therefore be exempt from GMO legislation and regulations.

Targeted integration of transgenes in plants via homologous recombination falls under the denominator of genetic modification. Plants that are produced with this technique must be considered as transgene. This implies, under the current legislation, that an environmental risk analysis will always have to be performed when a transgene is thus inserted.

Informative report

COGEM has observed that the development of new techniques demands greater clarity and perhaps also new interpretations of the current legislation and regulations regarding GMOs. The dividing line between what is a GMO and what is not is becoming increasingly more difficult to determine. Whether certain techniques are subject to the GMO legislation or not is principally a legal-political choice. Besides the technical-scientific arguments, social-ethical aspects can also play a role in this.

COGEM emphasises the economic importance of taking policy decisions in good time in connection with new techniques in biotechnology, as the decision as to whether certain techniques are subject to the legislation or not has important economic consequences.

COGEM is conscious of the European character of the legislation and regulations regarding GMOs and of the guarantee of co-existence and freedom of choice. Account must be taken of this European dimension when deciding whether to accommodate the new techniques under the GMO legislation or not.

COGEM points out that new technical developments complicate the enforcement of the European GMO legislation. As regards import, it will become increasingly difficult to detect mixing with non-registered GMOs. This shall raise the question of how the freedom of choice of the consumer can be guaranteed and whether the mandatory labelling of GMOs sufficiently guarantees this.

In its recent monitoring[1] on the ethical and social aspects of cisgenesis, COGEM has listed economic as well as other points of interest in case the government chooses to create possibilities for simplified admission procedures. These points of interest can also be important for deciding whether new techniques and their products are subject to the GMO legislation. COGEM points out that its advice to not accommodate some techniques under the GMO legislation is based on technical-scientific grounds. Not all in society will share this opinion. They may believe their freedom of choice to be limited if products, for which such techniques were used in the production process, are not designated as GMOs. This reasoning is strongly held in organic farming, which aspires to a process-driven and controlled form of agriculture. It is still unclear what the position and opinion of the consumer is. In deciding whether the products of certain techniques are subject to the GMO legislation or not, one point of consideration may be what the consumer expects with respect to labelling and the like. A consumer survey would perhaps provide more clarity on this.

Contents

1. Introduction

In selective plant breeding, varieties of plants are grown and selected which have the desired characteristics. These characteristics vary greatly from a higher yield or reduced sensitivity to disease and pests to improved product quality. To achieve this, the plants are crossed with each other and their progeny tested to see if they perform better than existing varieties. Plant breeding is a lengthy process. The time required from hybridisation to the introduction of a new variety is at least eight to ten years.

Biotechnology has given plant breeding an enormous boost. By applying new techniques originating from biotechnology, plant breeding has changed immensely over recent decades. Not having, for example, genetic markers for selection is unimaginable.

Genetic modification is only a small part of biotechnology. The stringent legislation, the high costs associated with compiling GMO acceptance files and the aversion of European consumers to genetically modified food are the reasons why Dutch breeding companies have little interest in genetic modification techniques. They sooner focus on techniques that make traditional breeding processes more efficient. However, some of these techniques are found at the cutting edge of what can and cannot be considered as genetic modification.

With this report, COGEM wants to bring the recent technical developments in biotechnology to the attention of the government. The commission wants to provide an insight into the current state of affairs in this field by offering insight into the possible applications of certain techniques, any risks and ambiguities in the legislation.

This report discusses six more or less new applications that will reach the commercial stage of application within the near future. For these applications, a sliding scale is discernible ranging from products that are clearly not transgenic because they contain no additions, changes or mutations in the genome, or changed properties, to plants that are clearly genetically modified. However, each of these new techniques raises questions on the interpretation of the GMO legislation. The answers to these questions are essential for further development of these techniques. With this report, COGEM aims to initiate the solution of the problems at stake.

2. Legislation and regulations

In the EU Directive 2001/18 "on the deliberate release into the environment of genetically modified organisms"[2] 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*".
This directive also states "*according to this definition: a) genetic modification occurs at least through the use of the techniques listed in Annex I A, part 1*".

The techniques referred to in this Annex are: "*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; and 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*."

These EU directives are implemented one to one in the legislation of the EU member states and are leading for determining which techniques, organisms and products are subject to the GMO legislation. However, two interpretations can be used. For on the one hand, it is argued that a GMO must contain changed genetic material and on the other hand, it is argued that genetic modification has occurred if certain techniques are used. In the definition of a GMO, a product-based approach is used (the end product is modified), while in the definition based on techniques a process-based approach is used (use is made of certain techniques in the production process). At the time of drawing up the legislation, this was not a problem; the use of the techniques referred to resulted in an organism with changed genetic material. Yet technological advances – as described in this report – now make it possible to use recombinant DNA techniques without an organism being created with changes to the genome.

This has resulted in the dilemma of which interpretation to use. Is an organism containing no genomic changes, which can thus in no way be distinguished from an unmodified organism, subject to the GMO legislation? Or should the process approach be used, with the underlying thought that the use of recombinant DNA techniques is associated with inherent safety risks that express themselves in the

products and about which consumers should at least be informed? Besides legal, technical-scientific and safety arguments, socio-ethical considerations also play a role in these questions.

3. Reverse Breeding

Characteristics of reverse breeding

The technique of 'reverse breeding' was developed by a breeding company.[3] The aim of the reverse breeding is to create parental lines of desired hybrid lines (not genetically modified). To achieve this, homozygote lines are created from the heterozygote plant.

This is done by inserting a gene in the heterozygote line (the hybrid) that suppresses recombination during meiosis. As a result, the haploid gametes of the genetically modified plant contain entirely non-recombinated chromosomes. These gametes can subsequently be used to produce plants. The plants that contain the transgenic sequence are selected out and only the non-genetically modified plants are used.

Using RNAi, genes can be silenced that facilitate recombination in meiosis. Various genes are involved in meiotic recombination. Genes that can be silenced are *asy1* or *sds*, which ensure that the homologous chromosomes pair in the first phase of meiosis. In addition, it is possible to turn off the *spo11-1* gene, which is responsible for the occurrence of double-strand breaks during recombination.[4] Furthermore, the *dmc1* gene can also be turned off, which facilitates the exchange of pieces of chromosomes during recombination.

To achieve the desired result, one copy of the RNAi transgene is inserted into the plant. In meiosis, only half of the haploid gametes will therefore contain the transgene, also because of the fact that the meiotic recombination is turned off. Then the chromosome number of the microspores formed is doubled. Microspores are the unripe pollen grains of a plant; they can form embryos in tissue cultures. Microspores are in principle haploid, but after the doubling of the chromosome number, fully disome, homozygote plants can be created from them. This technique is also called the doubled haploid technique.

Next the transgenic plants are removed. Only plants that do not contain the RNAi construct are used. These diploid, homozygote plants are used as parents for the reconstruction and seed production of the original heterozygote genotype. The end product of the reverse breeding technique is not transgenic, as it does not contain any foreign genetic material or other mutations in the genome.

Risks of reverse breeding

One of the most important characteristics of the reverse breeding technique is that the offspring are not transgenic. In the opinion of COGEM, a risk analysis, which has to be performed for transgenic plants, is therefore not needed for such

transgene-free plants that are made through reverse breeding. The plants do not have any new characteristics; nothing is added or changed in the genome of the plant. Reverse breeding gives rise to no new open reading frames, through which toxic or allergenic products could be formed. The plants are identical to the original parent lines of the original heterozygote line (the seed stock). COGEM considers the risks of reverse breeding products to humans and the environment or to food safety identical to the risks of ordinary breeding products.

Legislation applicable to reverse breeding

As remarked earlier, some argue that, according to European legislation and regulations (see note 2), a product should be recognised as a GMO if in its development process use is made of genetic modification. This would mean that offspring of a GMO should also be recognised as genetically modified, even when the gene concerned is no longer present in the genome of succeeding generations and no mutations or other changes are induced. This means that such plants are obliged to have a license and must be subjected to a thorough environmental and food safety risk analysis.

COGEM does not support this view and has also not been able to find a further basis for this interpretation of the European legislation. COGEM points out that the products of reverse breeding are not genetically modified and are identical to the 'natural parent lines' of the original seed stock. COGEM therefore believes that they are to be exempted from to GMO legislation.

COGEM recommends that plants that are acquired using the technique of reverse breeding should be handled as non-GMO.

COGEM points out that a problem of enforcement will arise if reverse breeding products are subjected to GMO legislation. Such products are in no way recognisable or detectable. The direct control of import from countries where reverse breeding products are not subjected to local GMO legislation is also not possible. Moreover, it makes it difficult to uphold the legislation in the field of traceability and labelling.

4. Agroinoculation

Characteristics of agroinoculation

The use of *Agrobacterium tumefaciens* to integrate genetic material into the plant genome is one of the most important methods for the production of genetically modified plants. The wild type bacterium causes neoplastic growths or galls in infected plants[5] by transfer of plasmid DNA (Ti-plasmid) into the genome of the plant (T-DNA). Expression of the Vir genes on the T-DNA in the plant cell leads to tumour growth.

When the tumour-inducing genes on the Ti-plasmid are replaced by genes that are responsible for a desired trait, these genes can be integrated into the plant. Plant cells with T-DNA stable integrated into the genome can be regenerated to fertile transgenic plants with the desired traits. Although infection with *A. tumefaciens* and transformation can occur in almost all parts of the plant, in practice, the parts and development stages of the plant that regenerate efficiently are chosen.

In agroinoculation, regeneration of transgenic plants is not the objective. The bacteria are injected using a hypodermic into certain tissue (such as the leaf), where the expression of the T-DNA occurs in the infected tissue.[6] Transfer of T-DNA to the nucleus of the plant cell does not need to lead to integration of the T-DNA in the genome or will remain limited to transfer and insertion into the genome of just a few cells of the injected tissue. It must be remarked that it is theoretically possible for the injected bacteria to spread through the plant and possibly transform cells elsewhere. Data that refute or confirm this possibility are largely missing.

In practical research and the breeding world, agroinoculation is principally used as a quick tool for testing plants for resistance or tolerance. Using agroinoculation, genes can be made to express themselves in the plant, allowing the response of the plant tissue to the proteins produced to be studied. Plants that appear to show the desired properties will subsequently be used and tested in the later breeding process.

Risks of offspring of agroinoculation

COGEM covered the question of whether plant seeds should be given a GMO-free status after agroinoculation before. COGEM came to the conclusion that offspring of agroinoculated plants should, in principle, be considered as not transgenic and that GMO legislation can therefore be considered as not applicable. However, at this moment one cannot entirely exclude the possibility

that offspring contain transgenic sequences after agroinoculation. It is indeed unlikely that transgenic DNA can be inserted in egg cells and gametes via internal transport of *A. tumefaciens*, but it is theoretically not ruled out. Experiments are described in which flower heads are dipped in an *A. tumefaciens* suspension ('floral dip') resulting in the insertion of T-DNA in the germ line cells.[7] In addition, in theory it cannot be ruled out that the outside of the seed is contaminated with the administered *A. tumefaciens*. Literature data on the (im)possibilities of unintended transformation of offspring and contaminations of seed with *A. tumefaciens* as a result of agroinoculation are absent.

COGEM consequently commissioned a research project to make missing knowledge aspects available. The results of this research are expected mid 2007. Based on the research report, COGEM will conclude what the risks associated with the offspring of agroinoculation are and whether a GMO-free status is defendable.

If it can be ruled out that *A. tumefaciens* gets into the offspring, COGEM will recommend assigning a GMO-free status to the offspring, in accordance with its previous recommendation. COGEM wants to point out now to the government that an alteration in the legislation for this technique may perhaps be useful.

COGEM recommends assigning a GMO-free status to the offspring of plants that have undergone an agroinoculation treatment, if it can be ruled out that *A. tumefaciens* gets into the offspring.

5. Gene silencing by DNA methylation

Characteristics of gene silencing by DNA methylation

In recent years, a lot of attention has been given to epigenetic effects in molecular genetics. Epigenetic effects refer to heritable changes in the function of genes that cannot be reversed by changing the DNA sequence. For the breeding industry, epigenetics is interesting because it offers the possibility of inducing effects in offspring, such as changed gene expression.

Numerous mechanisms underlie epigenetic effects that can occur within and between individuals and generations. The molecular mechanisms that shape the epigenetic code are mainly DNA methylation, histone modification such as acetylation, RNA interference and mechanisms based on chromatin (or chromatin changes).

RNA interference (RNAi) is an epigenetic mechanism of gene regulation. RNAi is an evolutionary conserved mechanism that ensures that genes are inactivated. RNAi uses double-stranded RNA and non-coding small RNAs as sequence specific regulators. Inactivation of genes, also called *gene silencing*, can occur in two ways: post-transcriptional and transcriptional.

Post-transcriptional gene silencing (PTGS) can be caused by the insertion of a transgene or double-stranded RNA, but also by a virus. In PTGS the mRNA formed is inactivated in the cytoplasm by homologous double-stranded RNA, which facilitates the breakdown of mRNA. The RNA is broken down after transcription; consequently no functional protein is formed. The RNAi mechanism is also active in the nucleus and involved there in RNA-dependent DNA methylation (RdDM). Due to this transcriptional gene silencing (TGS) can occur, which was first discovered in plants.[8] In general, it can be said that in eukaryotes DNA methylation plays an important role in gene expression, genomic organisation and stability, ‘genomic imprinting’ and developmental aspects.[9]

Genes in plants have been found, that are not expressed because of methylation of the promoter. Methylation is found everywhere on chromosomes and is seen as one of the most important control mechanisms of the cell. In areas where the DNA is strongly methylated the genes are generally inactive and areas with little methylation generally have active genes. These methylation patterns are meiotically stable and consequently heritable. In mammals, the epigenetic patterns are reprogrammed each generation. Consequently, these patterns are only heritable in mammals to a very limited degree.

Like the cytoplasmatic RNAi, RdDM requires double-stranded RNA that is broken down into small RNA molecules (21-24 nucleotides)[10]. When these small double-stranded RNA molecules have sequences that are homologous to the promoter sequences, they can effect methylation of the promoter. This facilitates transcriptional gene silencing.[11] Sijen *et al.* (2001)[12] demonstrated this process for the first time in an endogenous gene, of which the promoter was silenced.

The methylated status can continue in plants for a number of generations, even when the original RdDM-inducing transgene has disappeared as a result of hybridisation. This means that the offspring are non-transgenic plants, even though a gene has been silenced. Apparently the epigenetic effect is passed down over a number of generations during which the mechanism slowly loses power and dies out. This mechanism has sparked the interest of plant breeders and it could serve as an alternative to the 'traditional' RNAi. With 'traditional' RNAi, the RNAi transgene must always be present. In this way, it is possible for the breeder to produce a non-transgenic plant in which no changes or mutations are made to the genome but in which gene expression is influenced. Moreover, the application of RdDM promoter is comparable with that of regular RNAi. In other words, all the processes in which switching a gene off is good for production or consumption are looked at. Examples hereof are the silencing of fruit ripening genes, of a certain flower colour, of allergens and of oxidases that are involved in the browning of apples resulting from damage.[13]

Incidentally, at this moment, it is not possible to specifically turn off epigenetic effects, i.e. though 'switching off' genes is currently possible, 'switching on' 'inactivated' genes again is not currently possible.

Epigenetic effects are not exclusively the result of the above-mentioned technique or of genetic modification. They can also occur as a result of changed environmental conditions, in traditional breeding and as a result of spontaneous mutations resulting from the dynamic character of the genome. One of the causes of the variation in gene expression in hybrids with respect to gene expression in their parents can be epigenetic.[14, 15]

Risk policy and DNA methylation

This advice or monitoring report will not discuss the risks of DNA methylation or other epigenetic processes any further, as too little is still know about it. It is unclear how stable epigenetic changes are and how the mechanism of inheritance proceeds. Epigenetics is a new discipline and the possible application of epigenetic phenomena in plant breeding is still in its infancy. This report does not aim to give an exhaustive insight into the current state of affairs

surrounding epigenetics. COGEM has commissioned a research project into epigenetics.[16] The research report offers an overview of the current knowledge both in the field of plants and animals and of the possible applications.

Legislation surrounding epigenetics

At this time, it is unclear to what degree the application of epigenetic effects is subject to GMO legislation. If a transgene is present in the plant to induce the effect, there is no doubt that the GMO legislation is applicable. If one of the parent lines was genetically modified and one of its daughters carries the traits in question, it can be said that GMO legislation applies here. However, in other forms of induction of epigenetic effects, GMO legislation appears not to apply even though it concerns a (temporary) heritable effect.

COGEM observes that it is still too early at this time to make judgements on any environmental risks of epigenetic mutants. In addition, the question is to what degree such plants are subject to GMO legislation.

6. Grafting on genetically modified rootstock

Characteristics of grafting on GM rootstock

Grafting is a technique that has been used for centuries in plant breeding. In grafting, the bud-bearing part (the graft) of a plant is grafted onto the root-bearing part (the rootstock) of another plant. Particularly in fruit growing, grafting has been used from times immemorial. Rootstock is used, resulting in better growth control of dwarfed fruit trees or ones that are more resistant to diseases.

In recent years, the use of rootstock in the cultivation of vegetables is on the up and up. A large percentage of tomato, cucumber and aubergine crops are now grown on rootstock. Using rootstock shows to result in a substantial increase in the yield. Rootstock and upper stems are generally sold separately by the breeder. Grafting is specialist work that is performed by a cultivation company. The grower ultimately buys a grafted plant.

Nowadays, genetically modified rootstocks can be used that have been made, for example, fungus or virus resistant. An example of this is resistance against a virus that seriously damages cucumber, *Cucumber fruit mottle mosaic virus* (CFMMV). At this moment, no resistance genes are available against this soil pathogen. Genetically modified rootstocks have been produced that are made resistant to CFMMV by inserting a viral gene.[17] The upper stem of the resistant graft and consequently the fruit have, however, not been genetically modified.

Another application of grafting is currently also being experimented within laboratories. This technique concerns *short interfering RNA* (siRNA) molecules, which are made in the genetically modified rootstock. They are transported to the graft where they cause the desired effect.

siRNAs are oligonucleotides that are able to turn off a specific mRNA molecule. Grafting is an often-used technique to study the effects of iRNA under laboratory conditions. In most cases, the research is performed by grafting parts of tobacco plants. Now, it appears that application is at hand. Using this technique, protein production, for example, can be regulated in the upper stem and fruit without the upper stem requiring genetic modification. Another additional advantage is that numerous combinations of the GM rootstock are possible with various upper stems.

Risks of grafting on genetically modified rootstock

In the above described forms of grafting, the products of the upper stem are not genetically modified. However, the use of genetically modified rootstock is required to produce them.

COGEM points out that grafting with genetically modified rootstock must comply with the requirements of current legislation. By using this method of production, genetically modified plants – the rootstock – will be grown in the field. They must be evaluated according to the accepted methods of environmental risk analysis. The legislation could possibly be relaxed when the rootstock concerned does not produce any pollen, flowers or fruit. For some rootstock of, for example, woody crops (apples, pears, etc.), suckers may, however, grow from the rootstock. Such suckers could produce flowers and transgenic seeds. To prevent this, the wild shoots must be regularly removed. The possibility of spontaneous vegetative multiplication as potential environmental risk must also be looked at as this could lead to them running wild.

Regarding the upper stem and the products hereof, COGEM points out that they do not contain any transgenic sequences. However, transgenic proteins, hormones or siRNAs can be transported from the transgenic rootstock to the upper stem where they accumulate and cause an effect. Although the transgene is not present, the added characteristic can be expressed in the upper stem. The environmental risk that is possibly associated with this cannot be estimated in advance without having an insight into which specific protein it concerns. When a characteristic is created in the rootstock using an RNAi construct, it is possible that siRNAs are transported to the upper stem. A gene in the rootstock or a homologue of the gene in the graft can be silenced by this. It must be noted that the expression of genes is hindered in RNAi and the risk that a (toxic) protein is produced is very small.

A risk of spreading of a transgene by out-crossing is absent. Indeed the pollen originating from the flowers of the upper stem are not genetically modified. This also applies to the seeds.

Based on these considerations, COGEM recommends that a case-by-case approach is necessary and that it cannot be said that there are by definition no risks associated with upper stem (products) grafted on genetically modified rootstock to people and the environment.

Legislation applicable to grafting on genetically modified rootstock

As argued earlier, genetically modified rootstock are obliged to have a license and must be subjected to a full risk analysis. In the risk analysis, the possibility that molecules can be transported from the rootstock to the upper stem and accumulate there or cause an effect must also be looked at.

At this moment, it is unclear whether upper stems and the products of upper stems that have been grafted on genetically modified rootstock should be seen as genetically modified. This is a complex legal question, in which must be considered whether a graft is in a legal sense two different plants or one plant. In addition, the fact that the genetic material of the upper stem is not changed but that the upper stem displays possible changed characteristics or that a transgenic protein is present in the upper stem and fruits or seeds thereof plays a role. In such cases, it is imaginable that the European 'novel food' Regulation (EC) 258/97 applies.[18] In addition, it is also the question whether the products should be labelled as GMO.

For breeders and growers, it would be very beneficial if products of these grafted plants should have a GMO-free status. The legal ambiguities regarding the status of grafts are still undecided and have resulted in an impasse with respect to further development of this technique.

COGEM therefore recommends that the government gives a decision in the short term on the interpretation of the GMO legislation in connection with products of grafting on genetically modified rootstock. Here it concerns the commercial applications and market introductions. COGEM realises that this decision cannot be taken by the Dutch government but that it must be taken within a European context.

COGEM observes that it is desirable that a decision is taken about whether the products of grafts on genetically modified rootstock should, according to the current legislation, be seen as GMO or whether these products should receive a GMO-free status.

7. Oligonucleotides

Characteristics of applying oligonucleotides

In 2005, COGEM advised on the applications of oligonucleotides.[19] Oligonucleotides are short fragments of RNA and/or DNA that can be applied to people, animals or plants to regulate processes in the cell. Depending on the composition, oligonucleotides can bind to DNA, RNA or proteins and consequently regulate the expression of genes or change the DNA sequence. In the advice concerned, an overview was given of the various oligonucleotides and their applications.

Oligonucleotides are still little applied to plants. Until now, chimeric RNA/DNA oligonucleotides have been used to effect specific point mutations (chimeric surgery) in plants. Although these oligonucleotides are used in three plant species (maize, tobacco and rice), the same gene was the target of the mutations, encoding the enzyme acetolactate synthase (ALS) or, in maize, acetohydroxy acid synthase (AHAS).[20,21,22,23] Although the expectations regarding the application of chimeraplast mutagenesis were initially high, the chimeric RNA/DNA oligos appeared to be inefficient and the acquired results were difficult to reproduce. The expectations are therefore that this technique will be little used.

The COGEM advice on oligonucleotides also looked at the so-called third generation oligonucleotides, which owing to a chemical modification have both a high affinity for the target DNA and a reduced risk of being broken down by enzymes. They consequently stay in the cell longer and have an increased effectiveness. They are transferred via a transfection or electroporation to cells or protoplasts. Very promising results have been achieved in its application in animal and human systems, e.g. with 'locked' nucleic acids (LNA).[24,25] Currently, attempts are made to develop these types of oligonucleotides for plants.

A technique that may possibly be used in the near future in biotechnology is the combination of oligonucleotides and chemical mutagentia to effect a mutation at a specific site in the DNA. An example hereof is an oligonucleotide with a radioisotope attached to it, which binds to a specific piece of DNA and effects a double-stranded break when subjected to radiation.[26]

Moreover, the oligo provides for the specificity but does not itself produce any effect. This technique is a form of mutagenesis. But while in ‘traditional’ mutagenesis, numerous random mutations are caused in the genome by applying radiation or chemical mutagentia, a more specific change can be induced by

using mutagentia coupled with oligos. Indeed, unintended mutations through mismatch of the oligo or the presence of mutagens cannot be precluded.

Risks of oligonucleotides

In its 2005 advice on the risks of oligonucleotides, COGEM made a distinction between oligonucleotides that interact with RNA or proteins and oligonucleotides that interact with DNA. COGEM believes that the chance that sequence changes are induced in the genome when using oligonucleotides, which interact with RNA or proteins, is insignificantly small. At the same time, COGEM believes that oligonucleotides that interact with DNA can lead to sequence changes. Moreover, there is a very small chance that unintended sequence modifications can be caused as well as intended sequence modifications.

Legislation relating to oligonucleotides

The government has to date not made a decision regarding policy concerning oligonucleotides. COGEM's advisory report for 2005 regarding the applications of oligonucleotides endeavours to support this. COGEM asks special attention for techniques that use oligonucleotide to which chemical mutagentia are attached. COGEM foresees that such oligonucleotides with an attached EMS-molecule or other mutagens may be used in plant biotechnology in the near future, to effect a targeted (point) mutation in DNA. According to current GM legislation, organisms that are made using chemical mutagenesis are exempt from this legislation. COGEM believes that the coupling of an oligo does not change the character of the mutagen or the mutagen process. Granted, the increased specificity will reduce the risks associated with mutagenesis.

In COGEM's opinion, oligos linked to mutagens must be regarded as chemical mutagentia. COGEM also recommends treating organisms that are produced with these oligo-mutagentia the same as organisms made using 'traditional' mutagentia.

8. Targeted mutagenesis with homologous recombination

Characteristics of targeted mutagenesis with homologous recombination
In the current transformation techniques, the transgenes are inserted at a more or less random location in the genome. Efforts are therefore being made to develop methods to insert genes into the genome of the plant in a targeted way.

Integration of transgenes in the plant genome occurs via a process of so-called non-homologous recombination. In the eukaryote model system *Sachcaromyces cerevisiae* it was shown that non-homologous integration can be effected via the proteins that are involved in the cell in the repair of double-stranded DNA breaks via 'non-homologous end-joining' (NHEJ).[27] These NHEJ proteins are strongly conserved from yeast to plants and animals.

The random integration in the genome of transgenes results in an unpredictable structure of the transgene locus (such as the number of copies of the integrated gene, the co-integration of other so-called filler DNA and notable though unpredictable differences in the expression of the same transgene in various transformants, the so-called position effects). To prevent these effects, attempts are being made to develop methods with which the transgenes can be integrated in a targeted way in one single copy at a predetermined place in the genome. In addition, the focus is on two different methods, namely the use of a plant-based system for homologous recombination and the use of recombination systems (modified to the plant) for site-specific recombination.

The best-known example of a site-specific recombination system is the *Cre-lox* system originating from bacteriophage P1. In this regard, use is made of the enzyme *Cre* that catalyses recombination between two recombinase-binding sites, which are also called 'lox sites'.[28] In plant biotechnology, this system is used to remove antibiotic resistance genes and to make label-free plants, as well as for the site-specific integration of transgenes.[29,30]

The big benefit of using site-specific recombination for the integration of transgenes is that the same (molecular fully characterised) platform can be repeatedly used for the integration of 'single copy' transgenes. The structure and composition of the transgene locus can be determined beforehand and the expression of the transgene is predictable (apart from epigenetic effects).

The plant-based homologous recombination (HR) system can be used for the targeted integration of transgenes. To this end, transgenes are surrounded by sequences that are homologous (identical) to the sequences at the position in the genome where one wishes to insert the transgene. Such targeted integration works very well in yeast and in the embryonic stem cells of mice, but less efficiently in fungi and the somatic cells of animals and plants.[31,32]

All the same, transgenes with low efficiency (1 in 10^4 to 10^5 transformants) can, in principle, be integrated at the target in plants via homologous recombination.[29] However, the amount of work that is associated with the acquisition of such numbers of transformants makes the method unpractical for most crops. Still, research is being done to create a practical workable method for targeted integration, as such a method can also be used for the directed mutagenesis of plant-based genes. In the last case, a plant cell is transformed with a mutated version of (part of) a plant-based gene with the aim of integrating the mutation introduced into the gene construct into the plant-based gene. In this way, a plant-based gene can also be target activated via insertion. In combination with a site-specific recombination system, such as *Cre-lox*, the gene in the ultimate plant can be activated by the presence of just a small insertion (with stop codons).

The HR and NHEJ systems function in eukaryote cells like a repair system for double-stranded DNA breaks. Via HR, such breaks are removed and the chromosome is repaired using (preferably) the sister chromosome or the homologous chromosome as template. In NHEJ, the break is repaired by reconnecting the broken DNA strands. Often there are deletions or insertions at the place where the DNA break occurred.[33] Such double-stranded DNA breaks also form the entry site for the integration of transgenes.

In somatic cells of plants and animals, repair of double-stranded breaks preferably occurs via NHEJ.[32,33] This consequently means that the homologous recombination between foreign and chromosomal DNA only occurs in a few cells. Nevertheless, chromosomal changes can be effected in plants via HR. In this way, Terada *et al.* recently successfully genetically changed rice.[34]

Research is currently being performed on various fronts with the objective of increasing the efficiency of targeted integration and directed mutagenesis in plants using HR. Three different strategies are being followed: 1) the addition of components of homologous recombination systems from other organisms such as yeast; 2) the silencing of competitive recombination systems in plants such as NHEJ; 3) increased initiation of the recombination by specifically inserting double-stranded breaks in the DNA at the desired place.

Risks of targeted integration associated with homologous recombination

Targeted integration via homologous recombination will perhaps make it possible to safely produce genetically modified crops in the future. Transgenes integrate in the plant genome at random positions, while this method can be used to direct transgenes to a predetermined position and in addition effect targeted mutations in existing genes.

In homologous recombination, the creation of open reading frames that can cause possible damaging proteins can be prevented. Nevertheless, in targeted integration with homologous recombination, DNA is still inserted and the plant genome is changed. The plant acquires a new characteristic. In the opinion of COGEM potential risks linked to applying this method should be examined on a case-specific basis.

Legislation relating to targeted integration with homologous recombination

It leaves no doubt that targeted integration of transgenes via 'homologous recombination' and the resulting products are subject to GMO legislation and are obliged to have a license. In this regard, it must be remarked that the method can also be used to mutate plant-based genes without adding (almost) any new DNA. This will raise questions about why such products must undergo a full risk analysis pursuant to the GMO legislation, while other identical products that are produced in a different way (e.g. via chemical mutation) do not need to be evaluated. Although new techniques in biotechnology can differ considerably, the end result is sometimes almost the same.

With homologous recombination, a (point) mutation, for example, can be effected in a desired gene. This result does not differ from a mutation caused by an oligonucleotide or chemical mutagens. The difference is that with homologous recombination an almost identical piece of DNA is exchanged. With the exception of one mutation, this can only be an exact copy of the original DNA.

COGEM observes that with increasing refinement of techniques, - in which earlier technical-scientific objections were accounted for -, the principle "that there is an inherent risk if GMO techniques are applied during production" will come under increasing pressure.

9. Conclusions

COGEM has described a number of new biotechnology techniques that are found at the cutting-edge of genetic modification. Plant breeders, biotechnology companies and researchers have indicated that they are busy developing new plant varieties with these techniques. COGEM wants to emphasise that this report does not discuss theoretically possible techniques, but techniques for which people have commercial expectations. Breeders would like to bring to market the transgene-free offspring (via reverse breeding, agroinoculation) or transgene-free products (via grafting with genetically modified rootstock) produced by a number of these techniques. However, the further development of these techniques into commercial applications has stalled, as it is unclear whether the products are subject to GMO legislation.

COGEM realises that the Dutch government is bound by EU legislation. The Dutch government cannot unilaterally decide on exemption of certain techniques or products for commercial use. This is only possible via a joint decision from the EU member states. A unilateral interpretation of the Dutch government about whether an organism should be considered as GMO and obliged to have a license is undesirable. This could lead to a situation in which other member states could see the product as GMO and find the producer in violation upon import or production. This would also be in conflict with EU Directive 2001/18, which was drawn up with the objective of harmonising the internal market and avoiding such situations. COGEM therefore recommends that the Dutch government enters into consultations as quickly as possible with the other EU member states and the European Commission to come to a joint decision and judgement. COGEM intends that the information in this advice and monitoring report should be used to promote and support such consultations.

This document offers both recommendations and information on the (legal) dilemmas resulting from the advances in biotechnology and Considering the complex nature of the subject, COGEM has not written a separate advice and informative report, but has covered both aspects in one report. The most important remarks are briefly referred to below.

Advice

COGEM thinks the risks to man and the environment or to product food safety of reverse breeding, in which no trace of genetic modification can be found in the offspring or product, are the same as those to conventional breeding products. COGEM recommends that plants acquired with the technique of

reverse breeding should be considered as non-GMO or exempted from GMO legislation.

COGEM has earlier advised on the status of offspring of agroinoculated plants. COGEM believes that they are in principle not GMOs. However, at this moment it cannot entirely be ruled out that this offspring possesses unintended transgenic sequences after agroinoculation. At this moment there is insufficient data available to entirely rule out that seed is contaminated or gametes are transformed in agroinoculation. For this reason, COGEM is currently performing a research project to answer these questions. COGEM expects to be able to make a judgement on this in 2007.

The inducing of heritable effects in the function of genes without the sequence of the genome being changed is a promising application to the breeding world. However, it is still unclear how stable these so-called epigenetic changes are and how the mechanism of heredity proceeds. Direct applications of epigenetics without the plant and its offspring being genetically modified, are not expected in the near future. COGEM believes it is still too early to make any judgements on any environmental risks of epigenetic mutants. In addition, it is unclear whether epigenetic mutants fall within the legal scope of GMO legislation.

COGEM notes that the problems associated with grafting on genetically modified rootstock have been an issue for some years. It is unclear whether the non-modified upper stem and its products are subject to GMO legislation. COGEM points out that this is a legal-political question, about which the government must make a decision. With respect to the (environmental) risks, COGEM believes that it cannot be argued that there are by definition no risks to man and the environment associated with the upper stem (products) grafted on GM rootstock. Transgene proteins or siRNAs can be transported from the GM rootstock to the upper stem. For this reason, COGEM recommends a case-specific approach. The evaluation of the environmental risks can, for that matter, occur both within the scope of the acceptance of the GM rootstock alone and in an evaluation of the acceptance of the combination of lower and upper stem.

Earlier in a general sense, COGEM advised on the risks of oligonucleotides.[19] The commission asks for special attention for techniques in which oligonucleotides coupled to chemical mutagentia are used to effect targeted (point) mutations in the DNA. These could also be effected using ‘traditional’ mutagenesis. According to European legislation and regulations[2] mutagenesis is exempt from GMO legislation, although mutations are effected via mutagenesis

at many other places in the genome. The locations and effect of these are unknown. Using mutagentia coupled to oligonucleotides, better targeted mutations can be effected.

The coupling of a nucleotide sequence to chemical or radioactive mutagens does not change the character of the mutagen or the mutagenesis process. Because the oligo only has a positioning effect and does not interact with the genome or induce changes itself, COGEM believes that the oligo-mutagen complex can be considered as a 'normal' chemical substance or mutagen. For this reason, COGEM believes that directed mutagenesis using oligonucleotides is the same as 'traditional' mutagenesis and should therefore be exempted from GMO legislation and regulations. In addition, it must be noted that the risks to man and the environment are smaller with directed mutagenesis than with 'traditional' random mutagenesis.

Targeted integration of transgenes via homologous recombination, and the plants that are produced using this technique, undoubtedly fall under the denominator of genetic modification. This technique is included in the overview, as it overcomes a number of safety considerations of the current modification techniques. Using homologous recombination and related techniques, mutations can be inserted in the genes of plants in a targeted way. With this, unintended positioning effects such as the disruption of other plant genes or the creation of new chimeric reading frames are prevented. COGEM considers the risks associated with integrating transgenes using homologous recombination smaller than those associated with traditional transgenesis. Nonetheless, an environmental risk analysis will have to be performed when a transgene is inserted.

Dilemmas

COGEM points out that the development of the techniques outlined earlier, as well as future techniques, requires greater clarity and perhaps new interpretations of the current legislation and regulations concerning GMOs. The sharp defining line between what is and what is not a GMO has become increasingly more difficult to determine. Whether certain techniques are subject to the GMO legislation or not and whether a 'product' or 'process-based' interpretation of the legislation must be used is mainly a legal and political choice. Besides the technical-scientific arguments, socio-ethical aspects can also play a role.

COGEM wants to emphasise the economic importance of making policy decisions in good time in connection with new techniques in biotechnology. Indeed, companies and plant breeders partly determine their strategy based on

the conditions that the government creates. When the government decides to subject the discussed techniques or products of these techniques to the GMO guidelines, COGEM believes that it is realistic to expect that plant breeders and biotechnology companies will decide to discontinue working with the technique or in any case discontinue working with this technique in the Netherlands. The high costs associated with compiling a file for the acceptance of a genetically modified crop may make the companies decide not to proceed with the development of these techniques in the Netherlands or Europe. Therefore, COGEM points out that the decision about whether certain techniques are subject to the legislation or not also has economic consequences.

COGEM further notes that the development of increasingly refined techniques that can be used to quickly and efficiently achieve results, which can also be achieved via other methods that do not require a licence but are laborious, will increasingly raise questions about the need and reasons for performing risk analyses for certain GMOs.

COGEM points out that new technical developments will also influence the enforceability of the European GMO legislation. Some of the products of the techniques discussed in this report cannot be distinguished from products acquired in a conventional way. In this way, checking imported products, labelling, etc. becomes impossible. The issue of enforceability is for that matter not just a problem associated with these techniques. During import, it will also prove to be increasingly difficult to detect mixing with non-registered GMOs. This will raise the question of how the consumer's freedom of choice can be guaranteed and whether the mandatory labelling of GMOs can sufficiently guarantee this.

In its recent monitoring report[1] on the ethical and social aspects of cisgenesis, COGEM listed points of attention other than economic ones in the case that the government chooses to create opportunities for simplified acceptance procedures. These points of attention can also be important when deciding whether new techniques and their products are subject to GMO legislation.

The arguments are not all repeated here. But COGEM points out that its advice to not subject some techniques to the GMO legislation is based on technical-scientific grounds. Not everyone in society will share this opinion. Some will reject any form of recombinant DNA technique based on deontological arguments. They believe that their freedom of choice is limited if products for which such techniques are used in the production process are not recognised as GMO. This argumentation is widely supported in the organic

framing sector. The ‘process interpretation’ also fits the approach of organic farming, which strives for a process-driven and controlled type of farming.

Others shall oppose this and say that in the current breeding practice numerous DNA techniques are already used and that a distinction between these and the new GM techniques is difficult to make.

It is unclear what the position and opinion of the consumer is. When deciding whether the products of certain techniques are subject to the GMO legislation or not, a point of consideration can be what the consumer expects with respect to labelling and the like. Is the distinction between a product with modified genes and a product without modified genes that is the result of GM techniques important to the consumer? Consumer research on this would perhaps clarify this.

COGEM stresses the European character of the legislation and regulations regarding GMOs and of the guarantees of coexistence and freedom of choice. When deciding whether new techniques should be subjected to GMO legislation or not, this European dimension will have to be taken into account.

Overview table COGEM advice 'New techniques in biotechnology'

Technique		DNA insertion genetically separate from target gene(s)?	Heritable change?	Phenotypic change?	GMO?	Exempt from the legislation?	Detectable?
Reverse breeding		Yes	Before out-crossing: heritable change. Afterwards: not	No	Parent lines GMO. Offspring non-GMO	Parent lines not exempt.	Parent lines detectable. Offspring not.
Agroinoculation		Insertion disappeared	No	No	No	-	No
Epigenetic techniques	**RNAi, transcriptional; Gene silencing by methylation of promoter.**	Yes	Yes	Yes	Yes	No	Yes
	RNAi, transcriptional; Gene silencing by methylation of promoter. The insertion is 'out-crossed'.	Insertion disappeared	Yes, if the methylation is inherited.	Yes.	?	?	Yes
	RNAi, post-transcriptional. Building-in of DNA that forms RNAi. Gene silencing through breakdown of mRNA.	Yes	Yes.	Yes	Yes	No	Yes
	RNAi, post-transcriptional. Transient expression of DNA. Gene silencing through breakdown of mRNA.	Not applicable	No, insofar as the DNA is not built in.	Yes, where the DNA is transiently expressed, but otherwise not.	No, insofar as the DNA is not built-in or leads to heritable changes.	Yes	No
Grafting on genetically modified rootstock. Transgene protein or siRNA from rootstock to graft		Yes	Graft: No	Graft: Yes	?	?	Graft: not at DNA level
Targeted mutation using oligonucleotides		No	Yes	Yes	Yes	Yes	Yes
Directed mutagenesis by homologous recombination		Yes and no	Yes	Yes	Yes	No	Yes

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See also note 10.

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