# New Techniques in Agricultural Biotechnology in the Light of European GMO Directives

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# Background

- "New" Techniques (NTs) emerged in first decade of 21st century.
- Discussion on applicability of GMO Directive towards NTs.
- At the request of Competent Authorities under Directive 2001/18/EC, an Expert Working Group (WGNTs) was established (October 2007).
- WGNTs analysed a non-exhaustive list of techniques for which it was unclear whether they would result in a GMO.
- Working Group delivered a report in 2011.
- Report was envisaged to be considered by Authorities and Commission at policy level – as basis for future guidelines or new categorisation of techniques of genetic modification.

## “New techniques” considered by the WG

- Oligonucleotide Directed Mutagenesis (ODM)
- Zinc Finger Nuclease Technology (ZFN) (comprising ZFN-1, ZFN-2 and ZFN-3 as defined in this report )
- Cisgenesis (comprising Cisgenesis and Intragenesis)
- Grafting
- Agro-infiltration
- RNA-dependent DNA methylation (RdDM)
- Reverse Breeding
- Synthetic Genomics

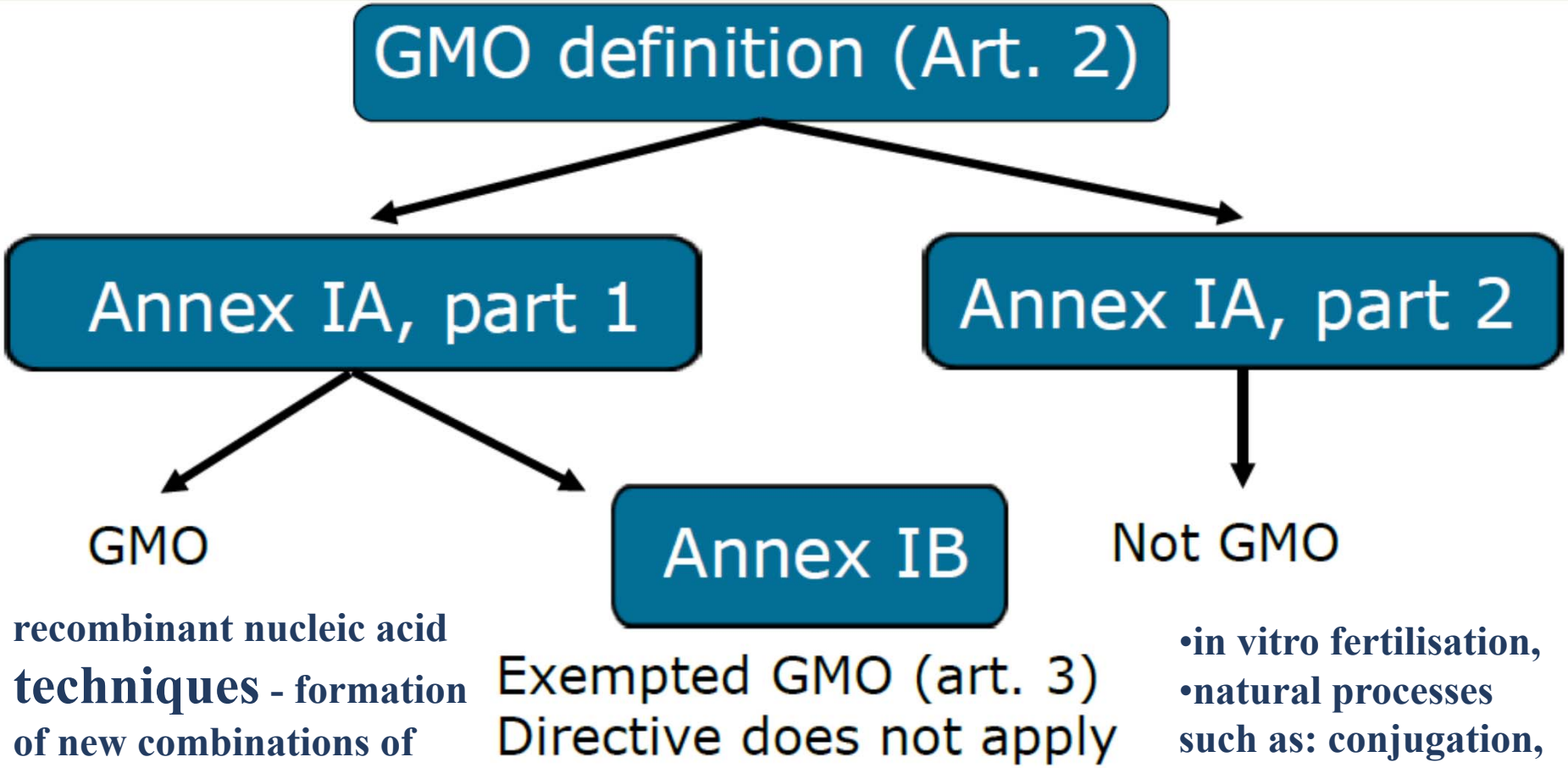
# Main Tasks of WGNTs

Do NTs result in genetic modification?

if so

Can they be exempted from the Directives?

# Directive 2001/18/EC

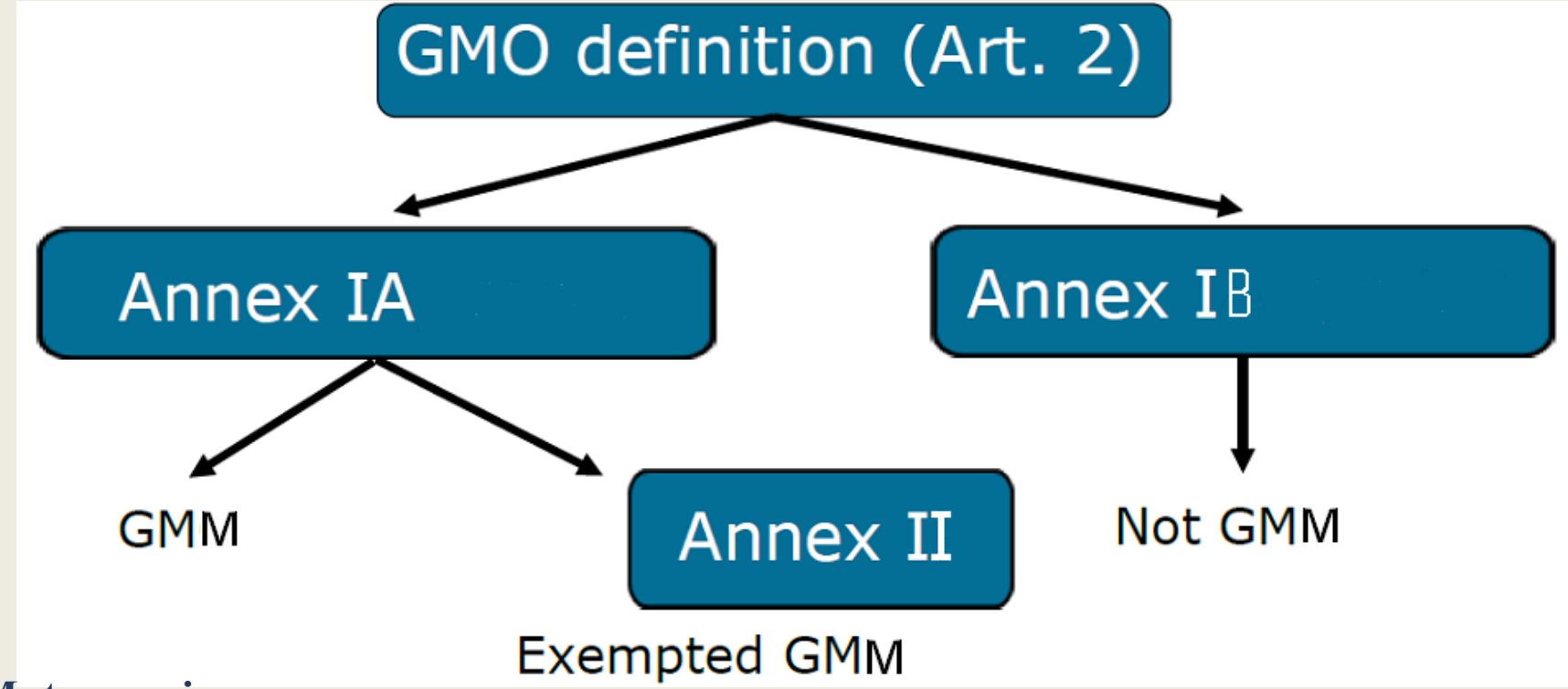



**recombinant nucleic acid techniques - formation of new combinations of genetic material prepared outside the organism**

**•mutagenesis,**
**•cell fusion (including protoplast fusion) of plant cells of organisms which can exchange genetic material through traditional breeding methods**

**•in vitro fertilisation,**
**•natural processes such as: conjugation, transduction, transformation,**
**•polyploidy induction**

# Directive 2009/41/EC




**•Mutagenesis.**

**•Cell fusion of prokaryotic species that exchange genetic material by known physiological processes.**

**•Cell fusion of cells of any eukaryotic species.**

**•Self-cloning consisting in the removal of nucleic acid sequences from a cell of an organism and reinsertion into cells of the same species or into cells of phylogenetically closely related species.**

**Self-cloning may include the use of recombinant vectors with an extended history of safe use in the particular micro-organisms.**

PROCESS BASED

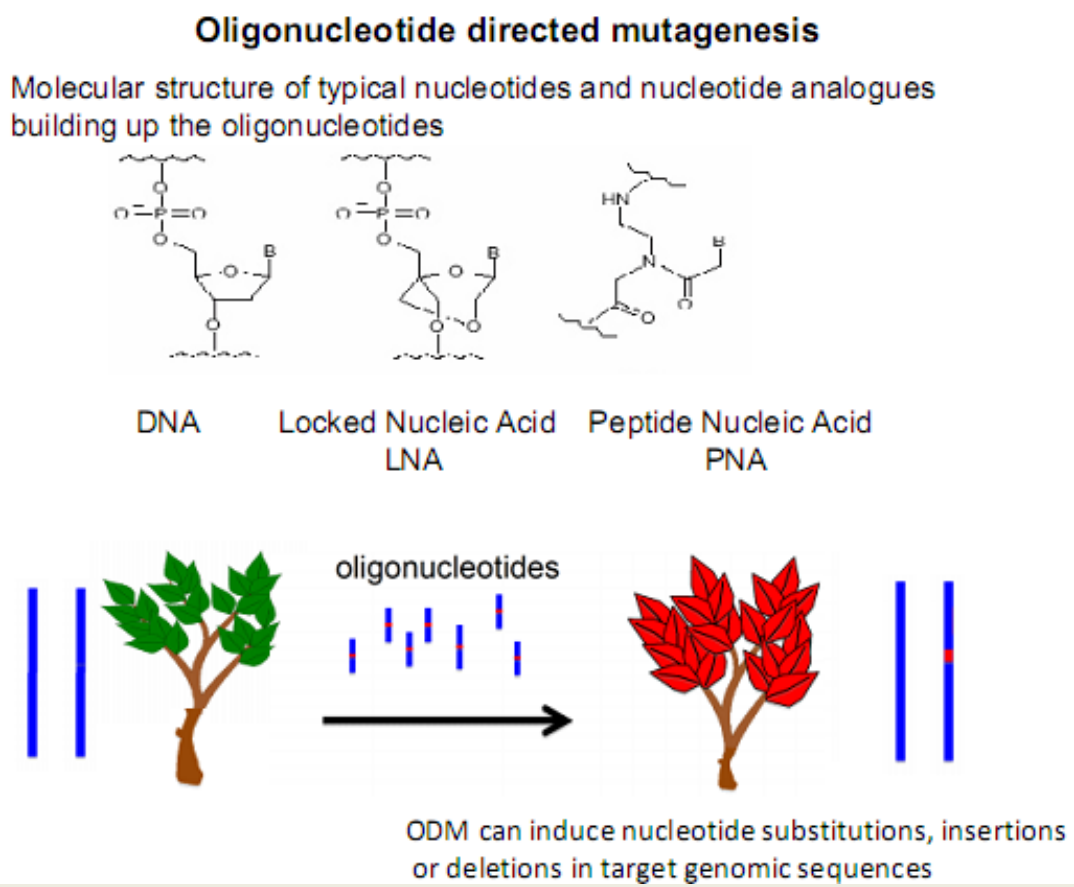
(Technique under regulation)

not

PRODUCT BASED LEGISLATION

Bases for interpretations

# Oligonucleotide-directed mutagenesis (ODM)




*The top row:* examples of typical molecules used for oligonucleotide-directed mutagenesis.

*The bottom row:* oligonucleotides above the black arrow; short sequences identical to plant genomic sequences are blue; red indicates a small sequence change. The resulting plant on the left (red) stably inherits the change to subsequent generations.

•Oligonucleotides of 20 to 100 nucleotides synthesised in order to share sequence with the target DNA sequence in the host genome, with the exception of the nucleotide(s) to be modified.

•Delivered to the cells by the common methods suitable for the different cell types.

•Results: induction of a point mutation; reversion of an existing mutation which may lead to changes in the expression of a gene.

# Controversial issues

**Nucleic acids:**

A majority of experts were of the view that oligonucleotides cannot be considered as recombinant nucleic acids in the sense of Directives.

For a minority of experts it is not possible to arrive at this conclusion.

## Coverage by GMO legislation

- **Directive 2001/18/EC/ Directive 2009/41/EC**

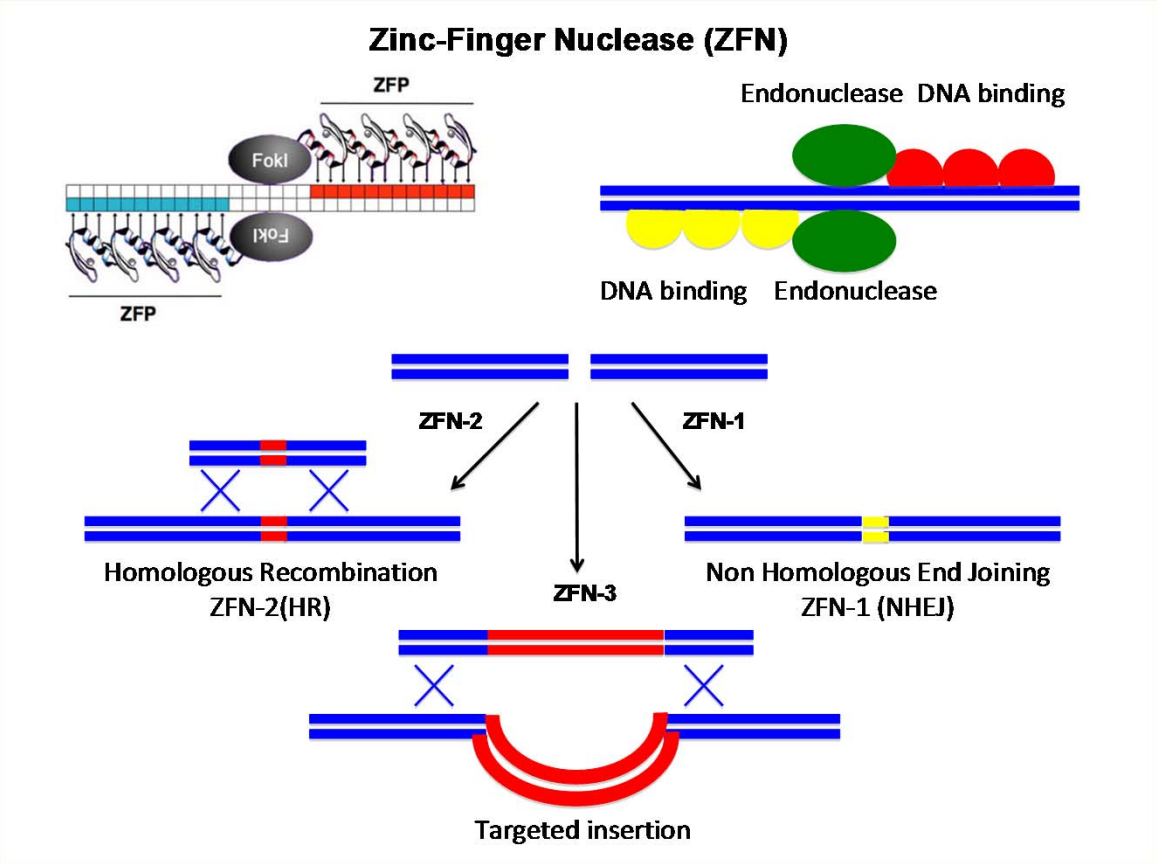
**Similarity to mutagenesis:**

ODM results in changes in organism that can be obtained with other forms of mutagenesis. ODM is expected to generate fewer unintentional changes or effects than those introduced into organisms by irradiation or chemical mutagenesis.

- **Detection**

Organisms developed through ODM cannot be distinguished at the molecular level from those developed through "conventional" mutation techniques (using chemicals or ionizing radiations) or through selection in natural populations.

# Zinc Finger Nuclease and related techniques




*Blue bars:* host double-strand DNA;

*Red bars:* heterologous double-strand DNA;

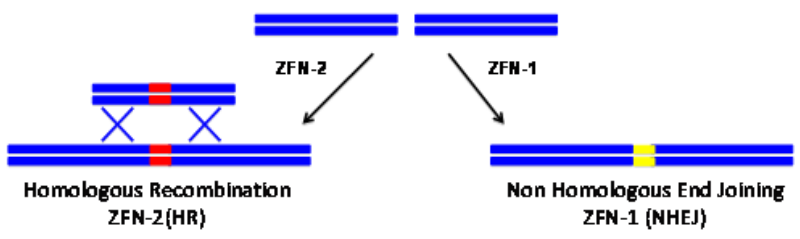
*FokI:* restriction endonuclease, the cleavage domains in ZFNs;

*ZFP:* Zinc-finger protein

•ZFNs are typically expressed **transiently** from a non-replicating vector (plasmid, virus) however, they may be delivered directly as proteins or as mRNA.

•In the cell, the ZFN proteins recognise the target DNA site as a heterodimer and generate a DSB. The DSBs are capable of triggering the cell's natural DNA-repair processes: homologous recombination and non-homologous end-joining, thus facilitating site-specific mutagenesis.

# Zinc Finger Nuclease-1 (ZFN-1) / Zinc Finger Nuclease-2 (ZFN-2)




A Zinc Finger Nuclease-1 (ZFN-1) generates site-specific **random** mutations (changes of single base pairs, short deletions and insertions) by non-homologous end-joining.

No repair template is provided to the cells together with the ZFN.

Result: in a single or a few base substitutions or small localized deletions or insertions. In the case of insertions, the inserted material is derived from the organism's own genome i.e. it is not exogenous.

B Zinc Finger Nuclease-2 (ZFN-2) generates site-specific the **desired** point mutation by DNA repair processes through homologous recombination.

A continuous stretch of DNA is delivered to the cells simultaneously with the ZFN. This template DNA is homologous to the targeted area, spanning a few kilo base pairs (kbp), and overlaps the region of the DSB. The template DNA contains the specific base pair alteration(s) to be introduced into the target DNA or chromosome.

Result: replacement of the original nucleotide sequence - one or a few bp.

# Controversial issues

**Transient presence/effect**:

Until recently, the approach has been to insert recombinant nucleic acids encoding ZFN into a vector. Provided the construct **does not replicate or integrate**, its presence in the cell is transient.

That recent technical developments allow the ZFN protein to be delivered directly into the cells, thus avoiding the need for DNA recombinant vectors.

**Heritable material**:

With regard to ZFN-2, there was a discussion as to whether oligonucleotides (in this case, the DNA template) constitute heritable material in the sense of the Directives, point on which experts did not agree.

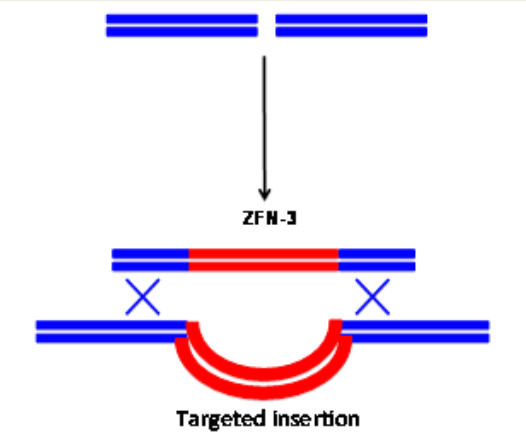
## Concluding remarks for regulation on the status of the technique

- The organisms resulting from ZFN-1/ZFN-2 are similar to organisms resulting from mutagenesis.

- There is a general agreement that the resulting organism from the use of ZNF-1/ZFN-2 is a GMO, but it should be excluded from the Directive.

## Detection

Organisms developed through ZFN-1/ZFN-2 cannot be distinguished at the molecular level from those developed through "conventional" mutation techniques (using chemicals or ionizing radiations) or selection from natural diversity.




# Zinc Finger Nuclease-3

Zinc Finger Nuclease-3 technique (ZFN-3) targets delivery of transgenes (insertions) by homologous recombination.

**Relevant issues for classification**

**Nucleic acids and recombinant nucleic acids**:

New combinations of genetic material are introduced into the cell when the donor DNA fragment is integrated into the recipient genome and is continuously propagated.

**Similarity to self-cloning:**

The technique may meet the criteria of self-cloning when:

- the donor DNA (or template of the synthetic DNA) originates from an organism of the same species as the recipient or from a phylogenetically closely related species;
- the recombinant vector has an extended history of safe use in the particular micro-organism;
- the resulting micro-organism is unlikely to cause disease to humans, animals or plants.

**Concluding remarks for regulation on the status of the technique**

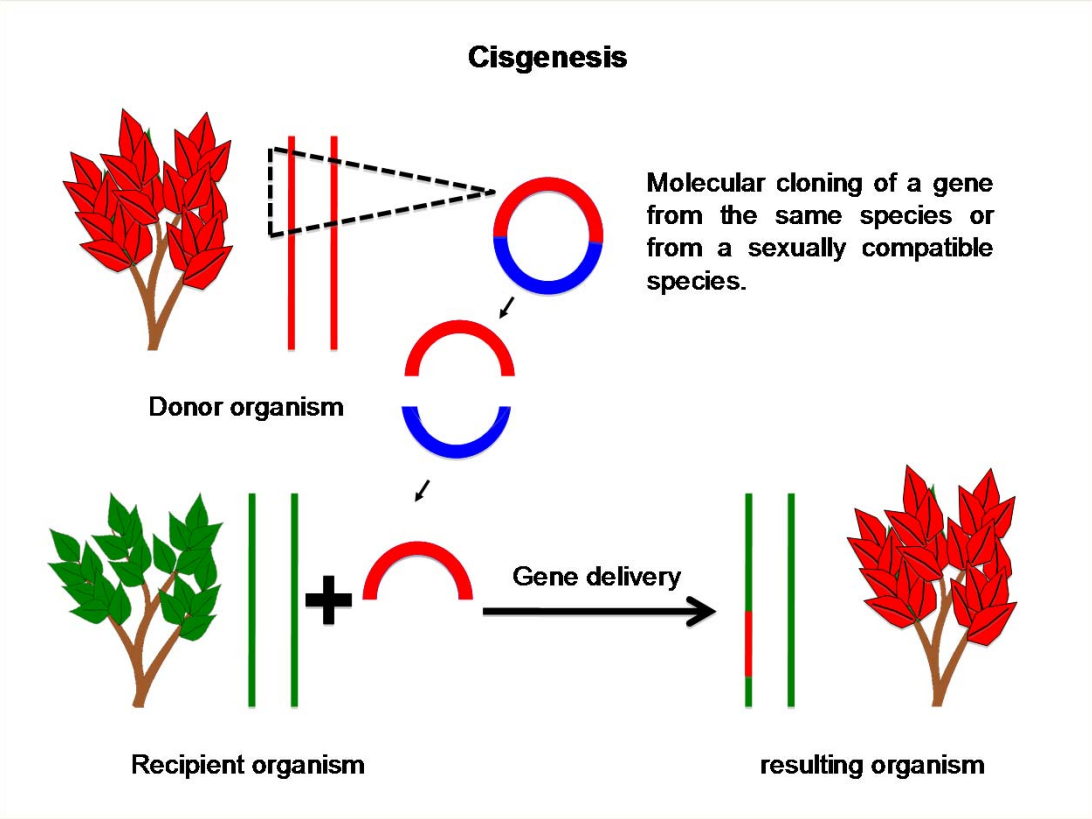
The ZFN-3 technique is generally within the scope of the Directives.

In some cases it could meet the criteria of self-cloning as described in Annex II, Part A of Directive 2009/41/EC and than it may be considered as falling outside the scope of Directive.

**Detection**

Detection and identification of organisms modified by ZFN-3 technology are possible through the amplification based methods (PCR) currently used for GMO detection, with the prerequisite that prior adequate DNA sequence information on the introduced modification is available.

# Cisgenesis and Intragenesis




*Blue*: nucleic acid from the vector;

*Red*: DNA sequences from the donor plant;

*Green*: DNA sequences from the recipient plant.

Donor and recipient organism belong to species which are sexually compatible.

Cisgenesis is genetic modification of a recipient organism with a gene (cisgene) from a crossable - sexually compatible – organism (same species or closely related species).

The gene includes its introns and its flanking native promoter and terminator in the normal sense orientation.

Sometimes the term cisgenesis is also used to describe an *Agrobacterium*-mediated transfer of a gene from a crossable - sexually compatible – plant. If T-DNA borders remain in the resulting organism after transformation, the technique is referred further in the text as cisgenesis with T-DNA borders.

Intragenesis is a genetic modification of a recipient organism that involves the insertion of a reorganised, full or partial coding region of a gene frequently combined with a promoter and/or terminator from another gene (intragene) of the same species or a crossable species. These may be arranged in a sense or antisense orientation compared to their orientation in the donor organism.

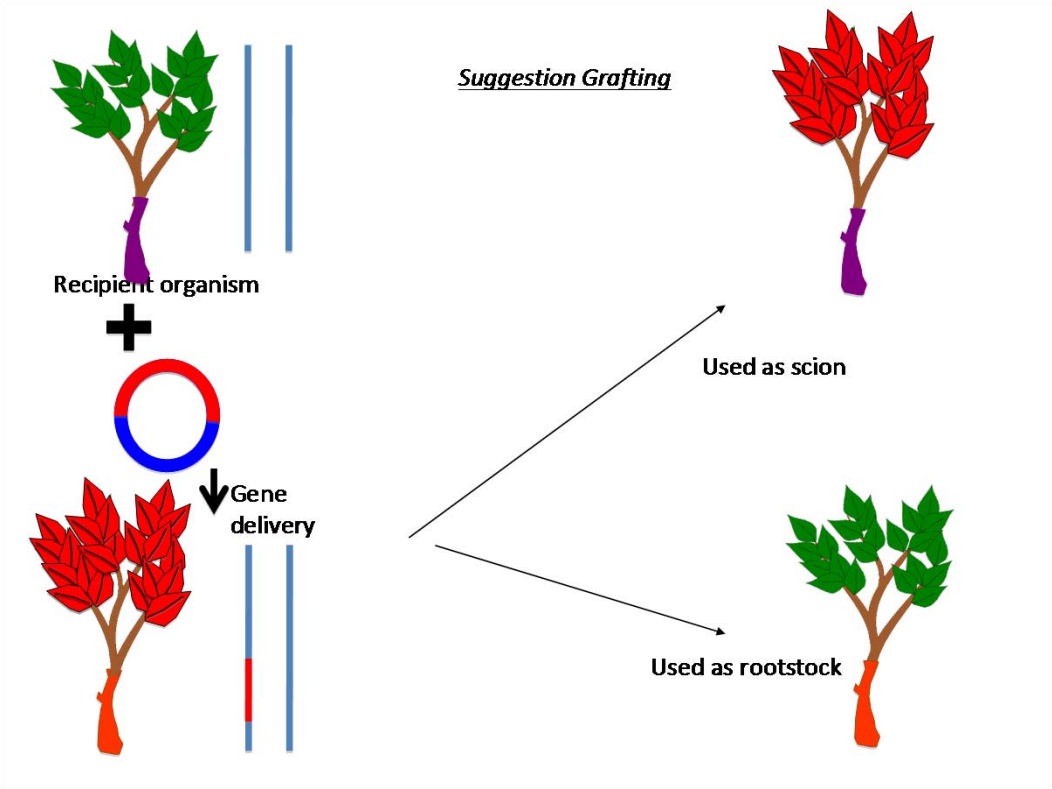
## Concluding remarks for regulation on the status of the technique

- The technique may in some cases meet the criteria of self-cloning as described in Annex II, Part A of Directive 2009/41/EC and thus is falling outside the scope of Directive 2009/41/EC. This included cisgenesis with T-DNA borders (ONLY with border sequences identical or highly similar (≥85% identity with sequences).
- However, self-cloning is not excluded from Directive 2001/18/EC.

## Detection

The introduction of cisgenes and intragenes into plants can be specifically detected if a sufficient part of the sequence of the insert as well of the adjacent sequence is known.

# Grafting




*Top left*: a conventionally bred plant line;

*Bottom left*: a transgenic plant line derived thereof;

*Right* two different chimera, where either the transgenic plant line or the conventionally bred plant line (bottom) are used as root stock or scion, respectively.

Two possibilities were considered (both - chimera plants):

Grafting a non-GM scion onto a GM rootstock;

Grafting a GM scion onto a non-GM rootstock.

Proteins and RNAs can be transported from the rootstock through the graft junction and into the scion and vice versa. This can affect the gene expression and phenotype of the upper (or lower) part of the plant. Although transport of macromolecules may occur, these *are not transmitted to the next generation* via the seeds as they do not lead to genetic modification.

**Coverage by GMO legislation**

There is a general agreement that the whole plant, which is a chimera, falls within the scope of Directive 2001/18/EC.

Where the fruit/seed/offspring are being considered and where a non-GM scion is grafted onto a GM rootstock, the resulting fruit/seeds/offspring derived from the scion do not fall under the scope of Directive 2001/18/EC.

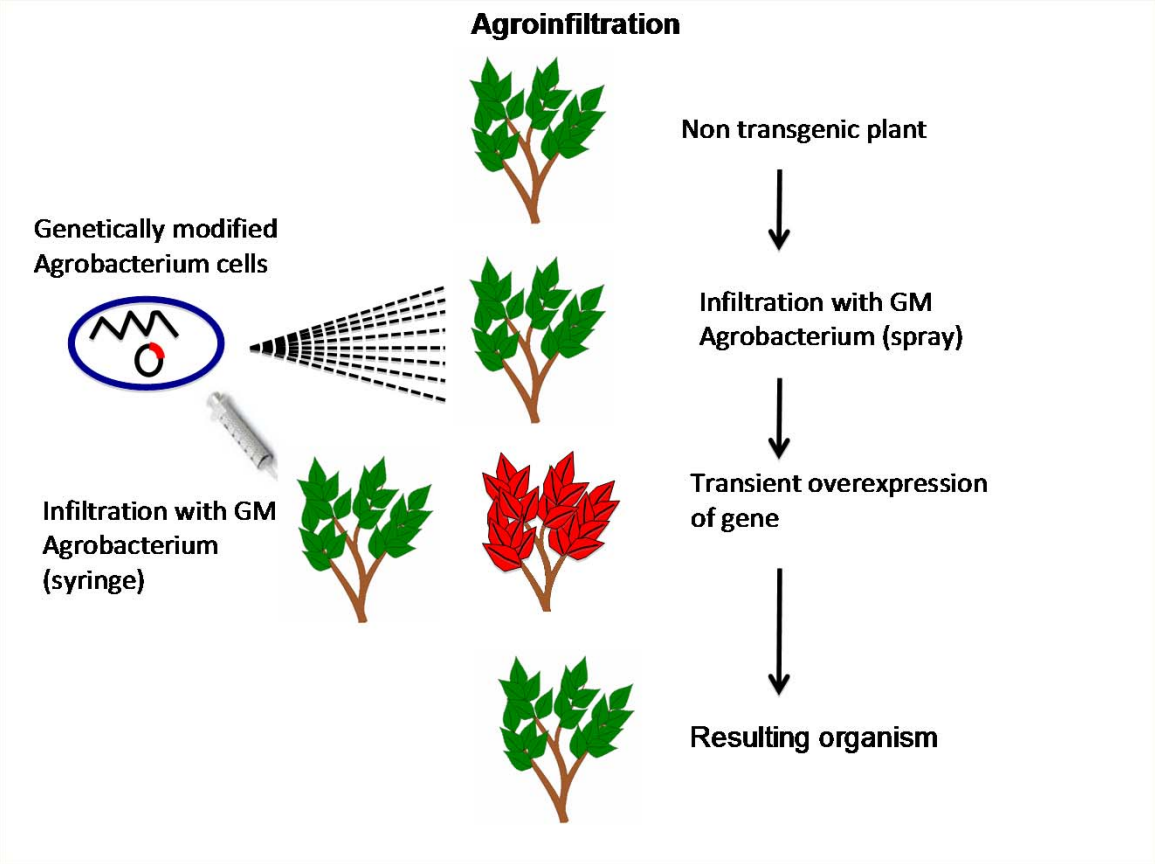
Where a GM scion is grafted onto a non-GM rootstock, the resulting fruit/seeds/offspring fall under the scope of Directive 2001/18/EC.

**Detection**

The transgenic part of the chimera can be specifically detected if a sufficient part of the sequence of the insert as well of the adjacent sequence is known.

As the DNA sequence of the non-GM scion is not modified, detection and identification of the GM rootstock on the basis of the harvested product (part of the non-GM scion) is currently not possible and is very unlikely to be developed in the near future.

# Agro-Infiltration




The syringe and the spray indicate two alternative ways of application.

The transient nature of agro-infiltration is reflected by the green plant at the bottom of the figure, where the red leaf colour has disappeared.

Note that **only very few, if any, plant cells at the site of infiltration are transformed**, thus the red plant here does not indicate that the plant is transgenic, but the transient expression of the infiltrated gene.

Plant tissues are infiltrated (*in vivo* or *ex vivo*) with a liquid suspension of *Agrobacterium* sp. containing a genetic construct in order to promote localised expression of a given genetic material. The benefits of agro-infiltration over stable transformation are speed, convenience, and the high level of expression usually reached.

In some *in vivo* applications the T-DNA may contain replicative material (in the form of either fully functional virus genome, or as “replicons" not able to spread within the plant).

# Application of the technique

Depending on whether or not the plant tissues contain germline cells/tissues, two types of agro-infiltration can be distinguished:

**Agro-infiltration "*sensu stricto*":**

Non-germline tissues (typically, leaf tissues) are agro-infiltrated in order to obtain localised expression.

**"Floral dip":**

Flowers or inflorescences containing germline cells are agro-infiltrated in order to obtain stable transformation of some embryos.

Nowadays this is the technique of choice to transform the model plant *Arabidopsis thaliana*, and it can also be used for other species in the same family (including rapeseed, cabbage, mustard etc).

Agro-infiltration takes place under conditions of containment. Progeny, if produced, is grown in a greenhouse and may be grown outdoors in some cases, for instance when agro-infiltration is used as a tool in the process of selecting commercial varieties with a given phenotype.

# Concluding remarks for regulation on the status of the technique

The recombinant bacterial vector and the progeny of the plants subjected to **floral dip** clearly fall within the scope of Directive 2009/41/EC (Annex I, Part A) and Directive 2001/18/EC (Annex IA, Part 1) respectively.

There are divergent opinions regarding the status of the agro-infiltrated plants with regard to Directive 2001/18/EC.

In situations where the agro-infiltrated plants produce a progeny, this progeny should be considered to fall outside the scope of Directive 2001/18/EC once the absence of a stable integration event is shown.

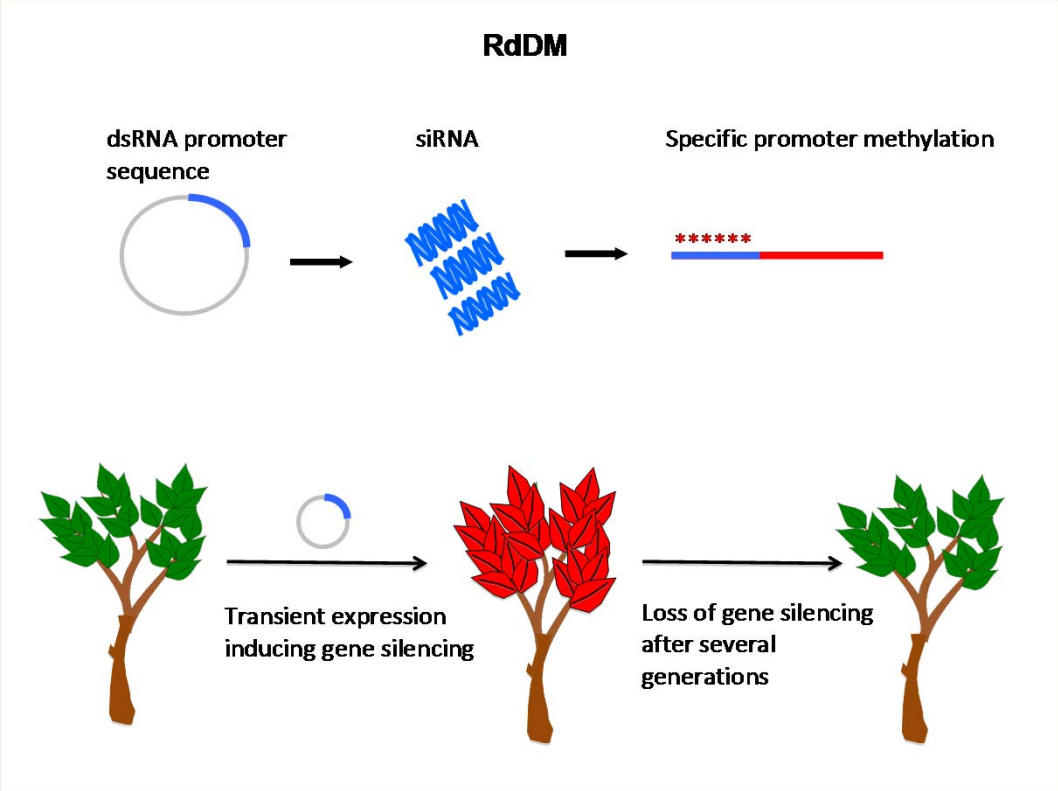
**Detection**

If the constructs introduced into plants by agro-infiltration are not replicated and/or integrated, their presence is transient and can be detected only in the agro-infiltrated plant itself.

These DNA fragments will not be transferred to the next generation so they cannot be detected or identified in the progeny plant and the products derived thereof.

Detection and identification of agroinfiltrated plants and progeny plants that contain stably inserted fragments as well as in the case of **floral dip**, the detection and identification are possible with the methods currently available for GMO detection (PCR), and also implies that adequate information needs to be available.

# RNA-dependent DNA methylation (RdDM)




siRNA can be introduced via a vector (*top left*), or introduced directly.

Specific methylation of plant genes is indicated by *asterisks* above the DNA sequence.

The *red plant* in the bottom indicates the (transient) silencing of plant. This does not necessarily mean that the plant is transgenic.

RdDM uses the effect of micro RNA (miRNA) or small/short interfering RNA (siRNA) to alter gene expression through methylation of specific DNA sequences (epigenetic change). The purpose could be to shut down expression of specific genes. This gene silencing obtained by the methylation can be inherited through some generations, but will eventually disappear.

In mammalian cells RdDM can be induced by direct introduction of double stranded RNAs (e.g. by liposomes or naked siRNA covalently conjugated with protein).
In plants RdDM is usually established by transformation with a DNA construct that encodes hairpin RNAs.

## Annex of Directive 2001/18/EC and Directive 2009/41/EC to which the technique may be attributed

The new methylation itself is not regulated by the Directives since methylation of nucleotides is not considered as an alteration of the genetic material in the sense of the Directives. Therefore, the resulting organisms are NOT covered by the Directives. This conclusion is reached even if the intermediate organisms are considered GMOs.

The resulting organism should logically be outside the scope of the Directives due to the fact that *the resulting organisms are comparable to organisms obtained with natural processes*.

The intermediate organisms do not fall under the definition of GMO of either Directives if the *RNA is directly delivered into the cell* without being able to replicate. The resulting organisms are thus not GMOs.

# Concluding remarks

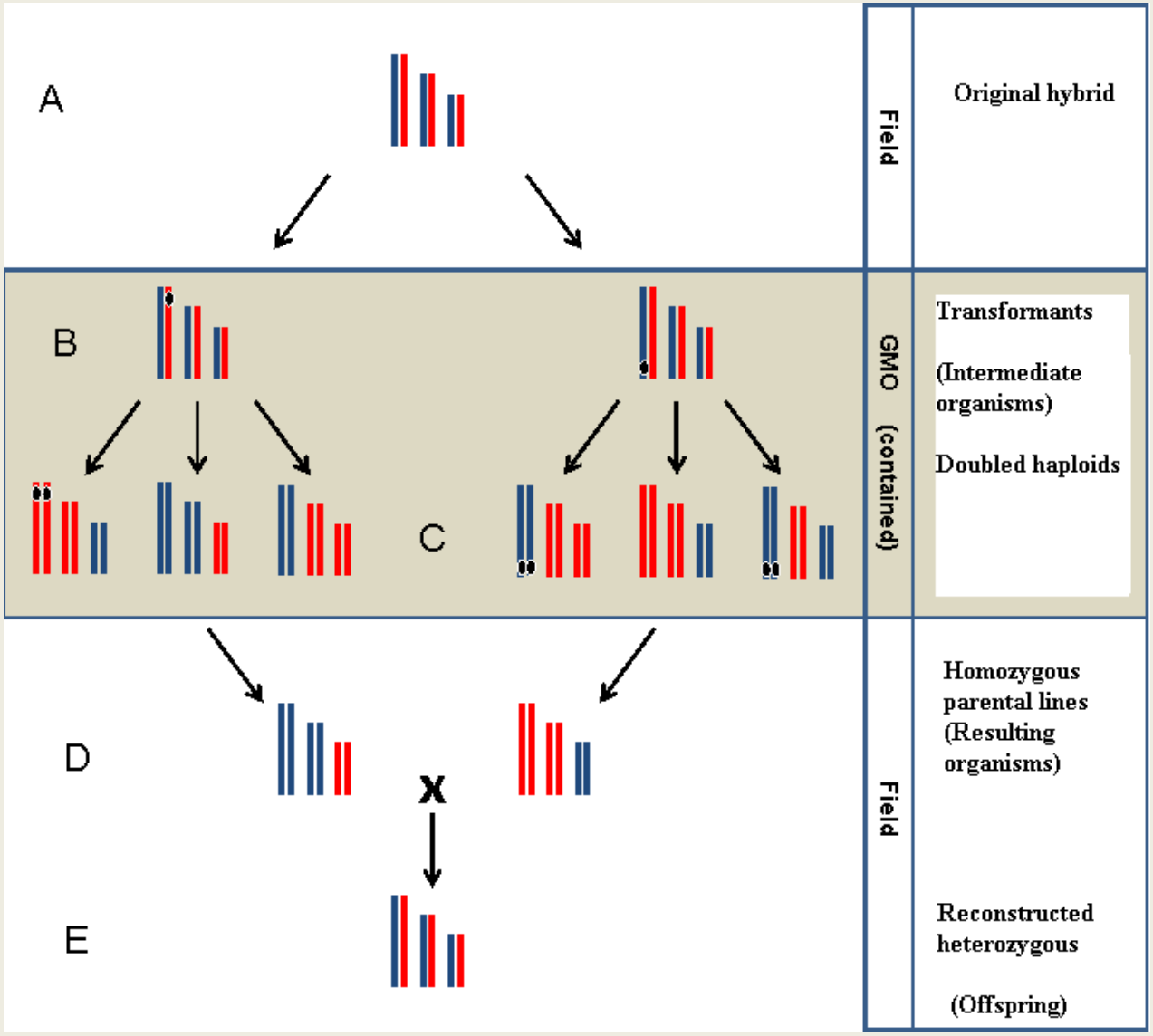
The current and future status of RdDM depends on the interpretation of the legislation as regards the use of intermediate organisms considered GMOs.

**Ones GMO, always GMO ???**

## Detection

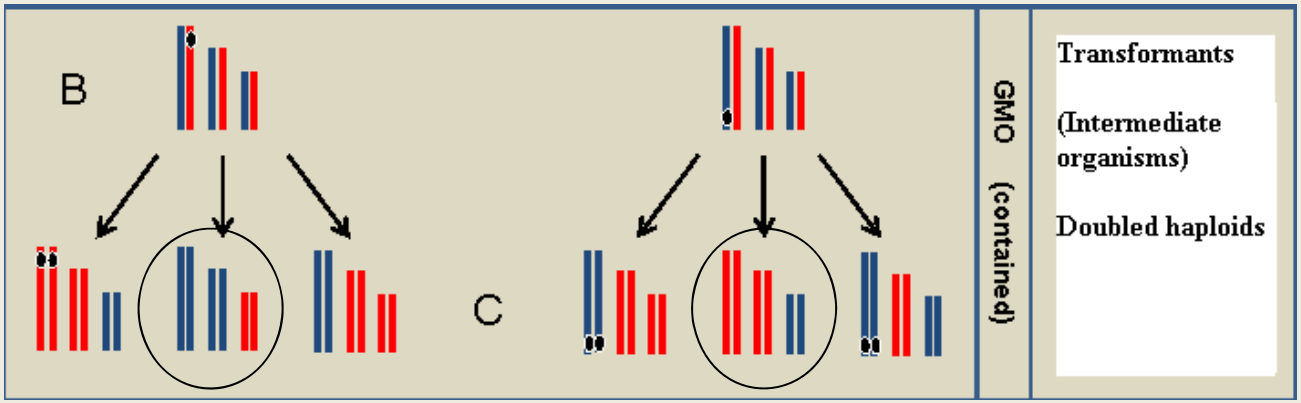
The characteristics (phenotype and methylation) can be used for identification only as long as the effect from earlier generations continues.

# Reverse breeding




An individual *heterozygous plant* is chosen for its elite quality (*A*), and, subsequently, *homozygous parental lines* are derived from this plant (*D*), which upon crossing, *can reconstitute the original genetic composition* of the selected heterozygous plant (Fig. 7, *E*) from which the lines were derived.

During reverse breeding, a genetic modification step is employed to suppress recombination during meiosis. However, the final heterozygous plants (and their homozygous parental lines) are non-transgenic (devoid of any new DNA).




## Steps to obtain the homozygous parental lines:

1) *Meiotic recombination* in the cells *is suppressed through RNAi* (RNA interference)-mediated down-regulation of genes involved in the meiotic recombination process. *The RNAi construct is integrated into one chromosome of the cells.*
2) Regeneration of these cells into plants. During flowering of this plant with suppressed recombination, haploid microspores (immature pollen grains) are formed.
3) The genomes of these haploid spores will subsequently be doubled upon specific treatments. The diploid microspores (*that do not contain the transgenic RNAi-constructs*) can eventually develop into embryos and subsequently into homozygous plants (so-called doubled haploids), the homozygous parental lines.

Result: Crossing appropriate pairs of those homozygous plants will create the desired heterozygous genotype. Using homozygous parental lines for the cross that do not contain the transgenic RNAi-constructs ensures *that the resulting final heterozygous plants are non-transgenic.*

## Concluding remarks for regulation on the status of the technique

Intermediate organism: The specific step where RNAi-mediated suppression of recombination is performed, gives rise to an intermediate organism falling within the scope of Directives.

The resulting organisms and their offspring are not GM and therefore may be considered as not within the scope of Directives 2001/18/EC and 2009/41/EC on the following grounds:

- The final heterozygous plants (and their homozygous parental lines) are non-transgenic.
- The resulting organisms and their offspring can be obtained by traditional breeding techniques.

**Detection**

Detection is not possible as the end-products of reverse breeding are free of genetic modification-related sequences.

# Synthetic Genomics

Synthetic genomics involves the synthesis of stretches of DNA molecules and their combination into functional larger synthetic DNA molecules which are then transferred into a recipient structure. The synthesis of building blocks enables the easy introduction of changes into the genetic material, including mutations (exchanges, deletions and insertions of specific nucleotides), gene fragments or complete genes including those without any natural template.

**Application of the technique**

As regards biotechnological applications, the construction of a minimal genome and its use as a basic framework to introduce biological parts could lead to the development of products such as biofuels, pharmaceutical products, cosmetics or products for bioremediation.

**Annex of Directive 2001/18/EC and Directive 2009/41/EC to which the technique may be attributed**

There are two possible interpretations as regards how the technique should be covered by the GMO legislation, depending on whether:

(a) the emphasis is on the resulting entity, which is considered to be a (micro-)organism. In this case, the technique falls under the scope of the Directives, which refers to techniques involving the direct introduction of heritable material prepared outside the organism.

(b) the emphasis is on the recipient (cell extracts or protocells), which is not considered as a (micro-)organism (see above). In this case, the technique falls outside the scope of the Directives;

# Results to date

- No legal analysis from EU Commission.
- No policy discussion among authorities for almost 10 years.
- Biotechnology develops further at a rapid pace. More techniques are emerging.
- Uncertainty and unclarity remains and increases.
- Continued discussion among *scientists* or *legal experts* without common and agreed understanding.
- Numerous attempts to initiate a policy debate were unsuccessful.

# Most recent developments

- The European Court of Justice has been requested to provide clarity regarding the interpretation of the Directive with regard to **mutagenesis**.
- The Scientific Advisory Mechanism (SAM) has provided a scientific report describing the technical characteristics of NTs at the request of the European Commission.

**Consistent policy approaches are required to improve the functioning of the internal market in EU and to ensure safety for human health and the environment.**

**THANK YOU FOR YOUR ATTENCION!**

**БЛАГОДАРЯ ЗА ВНИМАНИЕТО!**