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Briefing
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Genetic Engineering in Plants and the "New Breeding Techniques (NBTs)"

Inherent risks and the need to regulate

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Summary and conclusions

Over the last 5-10 years there have been rapid developments in genetic engineering techniques (genetic modification). Along with these has come the increasing ability to make deeper and more complex changes in the genetic makeup and metabolic pathways of living organisms. This has led to the emergence of two new fields of genetic engineering that overlap with each other: synthetic biology and the so-called New Breeding Techniques (NBTs).

As regards NBTs, it is of concern that many efforts seem designed primarily to avoid having to go through the regulatory process for GMOs, whilst choosing names that make it difficult for the public to see that genetic engineering (genetic modification) is being used. This goes alongside efforts to weaken the precautionary principle, which is there to guard against adopting technologies that are considered likely to bring negative impacts on human and/or environmental health in the future.

Currently there is a list of 7 "new" genetic engineering techniques before the European Commission, which is deciding whether or not the products of these techniques, when applied to plants, are covered by the EU GMO laws. Claims are being made by the industry either that they are not GMOs according to the current legal definition of a GMO, are made by techniques exempted from coverage, or that the final product, even if genetic engineering was used at some point during its development, does not contain GM material and so is no longer a GMO. The EC is currently working on the legal interpretation, as are many lawyers from industry and civil society. It is important to be aware - both in terms of legal interpretation and of risks - that some of theses techniques may also be used in combination with each other or the same technique may be used several times over in order to achieve the intended effect.

This briefing looks at these 7 techniques from the scientific rather than the legal perspective and aims to help readers to better understand the techniques and the inherent risks associated with them. Whilst examining the likely unintended effects it has become evident that all of the techniques claiming great precision are also found to have off-target effects with unpredictable consequences. In fact, so called precision is actually a very imprecise notion and does not equate to predictability.

In conclusion, the seven new genetic engineering techniques referred to as NBTs each bring their own set of risks and uncertainties. Whilst many of these are the same as with older GM techniques there are also serious additional concerns, such as the potential environmental and health impacts of RNA dependent DNA methylation (RdDM). Equally a new degree of uncertainty and risk of unintended effects arise from the use of gene editing techniques (ZFN and ODM as well as CRISPR and TALENs). This briefing concludes that there is a scientific case for classifying all these techniques as GM and regulating their use with as much rigour as previous and current GM techniques.

The 7 techniques under scrutiny are (following the EC's own titles)[1]:

1) Zinc Finger Nuclease Technology (ZFN-1/2/3)
2) Oligonucleotide Directed Mutagenesis (ODM)
3) Cisgenesis/Intragenesis
4) RNA-dependent DNA methylation (RdDM);
5) Grafting (onto a GMO rootstock);
6) Reverse Breeding (RB);
7) Agro-infiltration (both Agro-infiltration 'sensu stricto' & Agro-inoculation)

[1]http://ec.europa.eu/food/plant/gmo/legislation/plant_breeding/index_en.htm

An 8th technique was considered by the EC working group: *Synthetic genomics*. However it is generally regarded as a field within synthetic biology. Furthermore, no link to current plant breeding programs has been reported, though its application is being researched for example in microorganisms.

1) Zinc Finger Nucleases (ZFN) types -1, -2 and -3 (Gene–editing techniques)

ZFN techniques are genetic engineering techniques that aim for deliberate changes to the genetic make up and traits of an organism. They are also known as gene-editing techniques. Other gene editing techniques are now becoming prominent, but ZFN is the only one mentioned on the EU list.

The aim is to be able to change the sequence of the DNA, in order to delete, substitute or insert DNA sequences at pre-determined locations in the genome. In this way, the objectives are no different to any other engineering technique. In the case of the 'editing' techniques, this can mean small changes to 1-10 nucleotides[2] (ZFN-1 and 2), or large insertions of whole genes, including transgenes (ZFN-3).

For this purpose the DNA molecule first needs to be 'cut' at a specific location. ZFNs are proteins that are custom-designed and utilised for this purpose. The "zinc finger" (ZF) component can recognise a specific short stretch of DNA (9-12 bases) and the nuclease (N)[3] component will cut the DNA at that site. It requires two ZFNs – each to dock diagonally across the double stranded DNA – to cut through both strands. This DNA cut will then trigger one of the cell's two DNA repair mechanisms to stick the loose ends together again, with a number of possible outcomes.

<u>3 categories of ZFNs:</u>

- ZFN-1: small site-directed random DNA changes, which may be small deletions, substitutions or insertions of nucleotides. In this case the cell will 'repair' the break in a random fashion, using a repair mechanism called 'NHEJ' (non-homologous end joining).
- ZFN-2: small site-directed intended DNA changes, such as 'point mutations' (one nucleotide change). Here the repair will follow the instructions provided by a DNA 'template' that has been added (a stretch of DNA that has the same sequence as the target area but with one or two small alterations or a short insertion). Here the repair mechanism used is called 'HR' (homologous recombination).
- ZFN-3: large site-directed insertions of genes or regulatory sequences. In the genetic engineering process a DNA template will be added as in ZFN-2, but the template will also contain an additional long DNA sequence (eg one or more genes) for integration.

The gene for the specifically designed ZFNs will commonly be introduced into the plant through genetic engineering with standard GM transformation, making it a GMO at this stage. Once the ZFN proteins have been expressed and done their work, plant lines will be selected that do not carry the transgene for the ZFN proteins. Alternatively, in a declared effort to avoid being designated a GMO, plant virus expression systems have been developed where the ZFN gene is meant to stay within the viral expression system. The intention is that the ZFN transgene will not integrate into the plant's own DNA – and thus would not be passed on to future generations.

<u>Commercial Applications ZFN-1, 2</u>: The loss, change or insertion of a single nucleotide (point mutation) can be sufficient to change traits in a plant, such as: herbicide tolerance, male or female sterility, flower colour, delayed fruit ripening.

<u>Unintended changes and risks:</u>

- Off-target effects: ZFN technology is known for its non-specific binding to non-target DNA and thus result in a significant level of off-target mutations in the genome. These mutations can a) if in the coding sequence, result in changes of function of proteins, or b) if in regulatory sequences, result in changes in the expression of genes, such as increased presence of plant toxins, or absence of proteins important for nutrition, plant defence or disease resistance.
- Template DNA (ZFN-2 and 3) may integrate randomly into the genome, as do transgenic insertions, either as a whole or in parts, disrupting genes and regulatory sequences or potentially resulting in altered proteins. This may lead to a decrease in performance,

[2] *Nucleotides* are the building blocks of DNA and RNA, which is the genetic material also referred to as *nucleic acids*. The nucleotides are made up in DNA of A, C, G and T (Adenine, Cytosine, Guanine, Thymine); and in RNA of A, C, G and U (Adenine, Cytosine, Guanine, Uracil). It is the sequence of these letters that will determine which protein is being produced or which instruction is given.
[3] Nuclease: enzyme capable of cutting DNA.

heightened disease susceptibility, accumulation of toxins and residues, increase in allergens.

- Transformation and transfection[4] processes, including tissue culture[5], are used in the production of ZFN genetically modified plants. Such processes are known to lead to additional mutations (with risks as detailed in the bullet points above).[6]

Conclusion: All three ZFN techniques are genetic engineering techniques, aiming for deliberate changes to the genetic make up and traits of an organism. They are laboratory techniques. All three are prone to off-target effects due to the ZFN activity, as well as the effects of the genetic engineering processes, resulting in hundreds of mutations and unintended effects. Further, the plant's own repair mechanisms are not fully understood, giving rise to additional uncertainties. Due to the process, modifications and risks, ZFNs are GMOs and require full risk assessments.

Other gene-editing techniques:

There are several other gene-editing techniques, called TALENs, meganucleases and CRISPR/Cas.[7]

Though different in detail, they all consist of nucleases directed to specific DNA sequences where they will cut the DNA strand and evoke/trigger a natural repair system of the cell, as detailed above.

The same considerations that lead to ZFN techniques being classified as producing GMOs also apply to these gene-editing techniques. That is, they aim for deliberate changes to the genetic make up and traits of an organism; they are laboratory techniques and are prone to off-target effects, as well as unintended effects from the genetic engineering processes.

2) Oligonucleotide Directed Mutagenesis (ODM);

The aim is to create small and predesigned changes within very specific sites in genes, to either change the function of the gene product or to stop its production. For the purposes of ODM, an oligonucleotide[8] that is a short stretch of a single-stranded nucleic acids composed of a small number of nucleotides is synthetically produced.

It is designed to be almost identical to the DNA sequence of the target gene, except for 1-4 nucleotides. This will create a sequence mismatch when the oligonucleotide binds to the target gene, inducing a site-specific DNA change (mutation) once the cell's own DNA repair mechanism is triggered, preserving the sequence of the oligonucleotide rather than the original sequence.

Unintended changes and risks:

- Off-target effects: The oligonucleotide can bind to other DNA sites to which it is sufficiently similar, where it is likely to cause unintended mutations. These in turn can result in changes or loss of function of proteins, or changes in the expression of genes, leading to problems such as increased presence of plant toxins.
- The oligonucleotide can also integrate into the plant DNA, in a manner similar to transgenic insertions, disrupting genes and regulatory sequences or potentially resulting in altered proteins.
- The utilisation of tissue culture and GM type transformation or transfection[9] methods are known to lead to genome-wide unintended mutations.
- Near target site mutations have been observed in ODM derived GM organisms.

[4] *Transformation* of plant cells is the process of getting the external DNA into the cell and incorporated into the plants DNA. The term *transfection* of plants is more common when viruses are being used or when the external DNA is not meant to integrate.

[5] *Tissue culture* is the growing of plant cells in growth medium away from the plant. Through the use of nutrients, special compounds, enzymes and various growth hormones, cells can 1) reach the right stage for the transformation (insertion of the new gene sequence) and 2) then made to re-grow into a full plant. Tissue culture – especially the type used for plant transformation – is known to cause genome-wide mutations.

[6] See also Wilson et al. (2006), referenced in Background Information at the end.

[7] TALENs (transcription activator-like effector nucleases), MN (meganucleases) and CRISPR/Cas (clustered regularly interspaced short palindromic repeat system) – see also Agapito-Tenfen (2015) referenced in Background Information at the end.

[8] *Nucleotides* are the building blocks of DNA and RNA, the genetic material also referred to as *nucleic acids*. The nucleotides are made up in DNA of A, C, G and T (Adenine, Cytosine, Guanine, Thymine); and in RNA of A, C, G and U (Adenine, Cytosine, Guanine, Uracil).
Oligonucleotides are a stretch of genetic material (nucleic acid) and are commonly 20-200 nucleotides long; they may consist of DNA, RNA or nucleotide analogues, or any combination of those. They are commonly single stranded, but not always.

[9] see footnote 5 & 6

- Depending on the oligonucleotides used, there is a risk that the oligonucleotides may interfere with a cell's regulation of gene expression, by triggering the RNAi pathway[10], which can lead to gene silencing. This can manifest in heritable changes, that may last for many generations, and which depend on various factors that are not well understood. This may be more the case for oligonucleotides that contain RNA nucleotides.

Conclusion: ODM is a genetic engineering technique that can give rise to the same or to similar direct and indirect negative impacts as current GMOs, both due to the intended traits (eg herbicide tolerance, as performed by CIBUS for sulfonylurea herbicides in oilseed rape)[11], the processes and methods used and the potential integration of the oligonucleotides. It thus requires full risk assessment.

3) Cisgenesis and Intragenesis

Cisgenesis and Intragenesis are basically the same as transgenesis, but instead of sourcing the DNA sequence from totally different species or inventing a new synthetic DNA sequence, the sequence of the DNA inserted will be sourced from the same or closely related species, those with which the plant would, in theory at least, be able to interbreed. In 'Cisgenesis' the DNA inserted will have been made according to the exact sequence of a gene found in a related donor organism.[12] In 'Intragenesis' the inserted gene sequence is a composite, made up of sequences and elements from different genes of one or more closely related species; (see footnote for details)[13]

Unintended changes and risks:

- Whether or not the DNA sequences come from closely related species is irrelevant, the process of genetic engineering is the same, involving the same risks and unpredictabilities, as with transgenesis. There will be:
    - random integration of the transferred DNA, capable of disrupting another gene or interfering with the regulation of neighbouring genes (positional effects).
    - insertion-site mutations and genome-wide mutations resulting from the transformation processes, including the effects of tissue culture[14]. These can include deletions, rearrangements and multiplications of DNA sequences.
    - potential for gene silencing of the introduced gene or the plant's own genes if promoter sequences share high similarity (homology).
- re cisgenesis: The fact that the inserted gene comes from a related species is no guarantee that there are no unintended or unpredictable effects, as neither this particular gene nor its product would have been present before in this genetic context or position. Hence it may express in a different way from the way it did in the plant from which it is taken and/or interact (eg interfere) with wider gene regulation or metabolic pathways. This can give rise to altered behaviour and performance, higher susceptibility to disease, increased fitness and/or invasiveness, altered composition of signalling molecules[15], nutrients, toxins and allergens.
- re intragenesis: the DNA sequences assembled in such a gene will never have existed in this composition and in this regulatory context before. Their behaviour and interactions cannot be predicted simply by knowing the DNA sequence or by knowing that these sequences are derived from related organisms. Only a full analysis and strict assessment of the actual effects and impacts can provide answers.

Conclusions: with regard to risks and potential negative impacts, there is little to distinguish these techniques from transgenesis, therefore full molecular characterisation and full risk assessments, including comprehensive feeding trials for food and feed, are necessary.

[10] RNAi pathway. The RNA interference (RNAi) pathway is an internal cell process in which RNA molecules of various forms can, through a number of steps, lead to the silencing of genes.

[11] CIBUS is using ODM under the name of Rapid Trait Development System (RTDS™)

[12] The DNA inserted will not be taken directly from the donor organisms but rather be synthesised in vitro or amplified within the microorganism *E. coli*.

[13] e.g. the promoter, coding and terminal sequences may be derived from different genes and species.

[14] Tissue culture: see footnote 5

[15] *Signalling molecules* will transmit information between the cells and tissues of multicellular organisms. They can be simple molecules or complex proteins, such as growth hormones. Another category are the so-called *semio-chemicals*, that carry messages between different individuals either of the same species or between different species.

4) RNA-dependent DNA methylation (RdDM);

One aim is to obtain a new trait for a number of generations of seed, and to do so without changing any DNA sequences, ie the sequence of nucleotides, within the organism, in the hope of avoiding it being classified as GM. Instead, a process of RdDM[16] can be utilised within the cell to silence a specific gene, so there will be no gene product from that gene. This in turn can give rise to desired traits such as delayed fruit ripening, different coloured flowers, enhanced content of specific nutrients, male sterility.

RNA-directed DNA methylation (RdDM) is a process where RNA molecules direct the cell to add methyl groups (-CH_3 groups)[17] to certain nucleotides along a specific stretch of DNA in order to silence a gene. (for details see footnote)[18]

The methylation of the promoter region of a gene will stop the expression of that gene. Whilst such gene silencing is not a permanent alteration, it will be inherited for many generations. In plants it is thought to eventually fade, but this is not true for all organisms, e.g. in the nematode *C. elegans*. However the triggers for this reversal of the methylation are not known or understood.

How it works:

Any small double-stranded RNA with a sequence that matches the sequence of a stretch of DNA will initiate the methylation of these DNA sequences, and thus silence the associated gene. There are a number of ways to get specific sequences of double-stranded RNA into a cell, for example:
(a) genetically engineering the plant with a gene that will produce such an RNA (with an 'inverted'/reversed sequence) - intended for permanent or transient gene silencing.
(b) to have transient gene silencing, ie for a few generations only, the inserted gene can be removed (de-selected) by back-crossing in the breeding process.
(c) infection of plants with genetically engineered plant viruses (containing the targeted promoter sequence), which will result in the silencing of the targeted gene through methylation. ('Virus Induced Gene Silencing' (VIGS) – RdDM)
(d) spraying of plant with dsRNA (double stranded RNA).

Unintended changes and risks:

- off target effects: silencing of other genes, leading to altered traits, with potential negative impacts such as the production and accumulation of toxins and allergens, lowered nutrient content, disease susceptibility.
- the silencing of the target gene may not only stop the manufacture of the gene product (ie protein), but depending on the possible involvement of this protein in other pathways, may cause other unpredicted effects (often referred to as pleiotropic effects). Consequences may include anything that is linked to those pathways, eg growth factors, defence and signalling mechanisms, accumulation of compounds, etc.
- Specific to dsRNA: Depending on the methodology used, the presence of dsRNA molecules in the food chain and the environment may negatively impact other organisms exposed through ingestion or contact, for example in the case of sprays. They cay be passed down the food chain, and may be amplified and lead to the switching off of vital genes, which could have wide ecological and health consequences. This is the intended outcome, for example, with insecticidal dsRNAs produced in genetically engineered crop plants. This is a new and serious dimension of risk as compared to older GMOs.

Conclusion: The crucial question is not whether or not the final product (the plant) contains DNA sequences inserted through genetic engineering. It is rather that RdDM is a very new and little understood technology with potentially serious negative impacts both for consumption and the environment. It is a genetic engineering technology that, given the risks, needs full regulation and risk assessments.

5) Grafting: of non-GMO graft (scion)[19] on GMO rootstock; (and vice versa)

[16] RdDM is a form of RNA interference (RNAi).
[17] A methyl group (-CH_3 group) is made up of one carbon linked to three hydrogen atoms.
[18] In the case of higher organisms like plants and animals, only one of the four DNA nucleotides, the cytosine base, can be methylated. In lower organisms such as bacteria, the adenosine nucleotide can also be methylated.

[19] *Scion*: A young part of a plant (shoot, twig or bud) that has been cut for grafting.

Grafting (eg of fruit trees, grapevines, tomatoes)[20] is a way to combine the strength or desired traits of two organisms into one, without having to cross-breed them, eg rootstock for disease resistance and the graft or scion for fruit flavour. Though in combination a chimera (a single organism composed of genetically distinct cells), the graft and rootstock in themselves will largely keep their own genetic identities with regard to the basic sequence of their DNA.

The aim of using a GM rootstock is to create grafts that would benefit from the GM characteristics without being defined as GM or sharing the GM DNA, though, as a whole, the plants are GM.

Thus, strictly speaking, the tissue of the graft would not have been genetically engineered, while the rootstock has. Yet many of the molecules produced by the GM rootstock, whether proteins, certain types of RNA (eg: dsRNA), hormones, signalling or defence molecules, can spread throughout the whole of the chimeric plant.[21]

Unintended changes and risks:

- impact of the GM rootstock on the environment: genetic engineering processes, such as transformation and tissue culture (see footnote 1), are known to induce genome wide mutations, as well as insertion site mutations. These can lead to altered and unexpected traits, potentially with negative impacts on soil and environment. Positional effects of inserted genes (e.g.: affecting the expression of neighbouring genes) may equally lead to negative impacts.
- Compounds and metabolites produced by the GM rootstock will be present in the graft and its products (eg in fruit) and may alter the composition of the fruit/product, which in turn may alter the nutrient, allergen or toxin composition.
- If RNAi (RNA interference) methodology has been used in the GM rootstock, the gene silencing active in the DNA of the rootstocks could transfer to the DNA of the graft via the movement of small RNA molecules from the rootstock into the graft. This may silence genes in the graft and alter its traits and vice versa.

Conclusion: To obtain the GM chimeric plant, by definition, requires genetic engineering, and the risks arising are due to the genetic engineering (the inserted sequence, its location and the transformation processes). The fact that the graft does not have any of the genetically modified DNA does not necessarily reduce the risks to the environment, ecosystems and/or human and animal health. As molecules/compounds can travel between rootstock and graft, affecting the behaviour and molecular composition of the graft, both the plant as a whole and the graft and its products need to be defined as GM and fully assessed and regulated. This is particularly the case as the processes and interaction between rootstock and grafts are still poorly understood.

6) Reverse breeding (RB)

RB is a GM technology intended to reconstitute genetically uniform and pure (homozygous) parental lines from an existing hybrid whose parental lines are no longer available or no longer exist. A major hurdle in this is that, every time gametes (reproductive cells) are produced, the chromosomes previously acquired from the parental lines swap information during the genetic recombination stage[22], thus mixing the DNA. To avoid this, the selected hybrid seed is genetically engineered to suppress genetic recombination (using RNAi). With the help of tissue culture, individual resulting gametes are used to reconstitute plants with two sets of the same chromosomes (called 'double haploid'). At a later stage the GM gene is deselected and parental lines chosen that – in combination – will give rise to the envisaged hybrid.

Unintended changes and risks:

- As the same genetic engineering processes are used, both to insert genes and to reconstitute plants through tissue culture, the same risks and unpredictable outcomes

[20] *Grafting*: has been common for woody plants for over 2000 years, and is often used for fruit trees, roses and grapevine. Grafting of vegetables is more recent, mostly used for tomatoes and watermelon, but also cucumbers and eggplant.

[21] the transport is especially via the *phloem*, which is a type of vessel tissue that transports water, food and nutrients up and down (ie in both directions) to growing parts of the plant.

[22] *Meiosis* is a process of cell division resulting in gametes, i.e. the male or female reproductive cells, each containing half the number of chromosomes (*haploid*) as compared to the ordinary plant cells (*diploid*), that has two sets of chromosomes, one from each parent.

are possible as with other GM. There will usually be:
  - insertion site and genome wide mutations (eg deletions, rearrangements, multiplications) resulting from the transformation processes, including tissue culture, with unpredictable consequences that could lead to altered performance and disease susceptibility, accumulation of toxins, increased production of allergens, changes in nutritional composition.
  - The vast majority of these mutations would remain present in the reconstituted parental lines even if the GM gene itself is deselected and with it the mutations most closely associated with the insertion site itself.
- The GM gene silencing method of RNAi may lead to non-target gene silencing of other genes, effects that will be maintained for many generations of seed. Thus tests for performance and compositional analysis will need to be carried out, followed by full risk assessments. These need to take place before initial planting, but also several generations later, once the intended and unintended gene silencing has faded and it should include feeding trials.
- Functional components or full sequences of the GE gene may have integrated themselves elsewhere in addition to the primary insertion. They may thus not be removed in the de-selection process, leaving them potentially still able to initiate gene silencing in the target region or in off-target areas.

Conclusion: Parental lines as well as the combined new hybrids need to be tested for the presence of GE sequences as well as for unintended effects due to off-target gene silencing and transformation induced mutations, which have the potential to, for example, result in altered performance and disease susceptibility, accumulation of toxins, increased production of allergens, changes in nutritional composition. Full risk assessments are required.

7) Agro-infiltration: Agro-infiltration 'sensu stricto' & Agro-infection

This method involves two distinct technologies. It is not intended to result in specific GM genes being stably inserted and integrated into a plant genome, but rather for such genes to be present within the plant cell transiently, for a maximum of just one generation.

To this end, genes either coding for specific proteins or for RNAs to interfere with the plant's own genes (eg via RNAi) are engineered into the plasmid[23] of *Agrobacterium tumefaciens*.[24] A solution of such Agrobacteria or their plasmids is then used to treat specific tissues of living plants (eg leaves) so as to have the plasmids with the GM genes delivered to the cells in that tissue, where these genes will be expressed in the specific RNA.

The aims may be to: test potential transgenes; study the function of the plant's own genes (eg through gene silencing via RNAi); express and produce high value proteins in plants (eg pharmaceuticals); produce plants, seeds, hybrids with altered traits through RdDM (RNA dependent DNA methylation – see section 4); or use as a delivery system for other GM-based NBT tools, such as site directed nucleases.

Two distinct technologies:

Agro-infiltration 'sensu stricto' (ie in the narrowest meaning): The intention is to keep the gene expression and effect localised, thus the genetic construct prepared and used is not expected to replicate in the receiving cell.

Agro-infection: The intention is to spread the specific GM gene throughout the whole plant into almost all the tissues, but without integrating the gene into the plant's DNA. For this purpose, in addition to the chosen gene, the gene construct contains a viral vector sequence in order to replicate the construct in all infected cells. The gene for the RNA is meant to be expressed from its location on the vector, ie not from a location on the plant's DNA.

Unintended changes and risks:

- Though applied locally, the gene construct can spread throughout the plant, due to the agrobacteria and/or the viral vector sequences used. Although meant to be transient, the genetic material may become integrated into the plant's DNA, including

[23] A *plasmid* is a circular ring of DNA in a bacterial cell that can replicate independently of the chromosomes and can be passed on to other bacteria. Here it contains the transgenes.

[24] Using *Agrobacterium* as a gene shuttle is one of the major means of performing GM).

reproductive tissue, thus unintentionally giving rise to GMOs and to GM progeny.
- Integration may happen at random places within the genome and may also involve any of the DNA sequences introduced, including vector DNA. Disruption of genes due to positional effects or due to sequences present in the gene construct could give rise to negative impacts on plant performance, environment and biodiversity, or on its safety as food.
- Accidental release of genetically engineered Agrobacteria into the environment could occur (either due to the spread of and contamination from infiltrated plant material that has been discarded or removed, or simply through spillage, eg from lab, greenhouse or test plots). This in turn could give rise to adverse effects if the gene constructs get transferred to other plants or to microorganisms.
- Replication can occur at levels too low to detect for long periods of time, increasing the chance for either integration or mutation that makes the DNA stably heritable.

Conclusion: plants subjected to Agro-infiltration (including agro-infection), along with any of their parts and products as well as their progeny need to be tested for the presence of DNA sequences from the vector and/or the gene construct, as well as for the presence and effects of gene silencing, if that was the initial aim of the agro-infiltration.

For background information and further reading, four of the most important references:

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http://www.umweltbundesamt.at/fileadmin/site/publikationen/REP0477.pdf

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