Bundesamt für Verbraucherschutz und Lebensmittelsicherheit

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Position statement of the ZKBS
on new plant breeding techniques

I. Grounds

In the European Union (EU), the handling of genetically modified organisms (GMOs) is legally regulated by Directive 2001/18/EC on the deliberate release into the environment of genetically modified organisms and by Directive 2009/41/EC on the contained use of genetically modified micro-organisms. In Germany, these Directives are essentially transposed into national law through the *Gentechnikgesetz*, GenTG [German Genetic Engineering Act]. Recently, new molecular biology techniques have been developed for which it must be clarified whether the resulting organisms are genetically modified within the meaning of this legislation.

For this reason, at the request of the Committees of Competent Authorities, the New Techniques Working Group (NTWG) was established in the EU, to which each EU Member State was invited to delegate two experts. The NTWG, with the organisational support of the European Commission, described new molecular biology techniques and examined whether or not they give rise to GMOs within the meaning of Directives 2001/18/EC and 2009/41/EC. The task of the working group was to make their findings available to the Competent Authorities of the EU States as technical advice with their report. In December 2011 the NTWG submitted a final report to the European Commission. Inquiries from federal state authorities have now prompted the ZKBS to issue a statement on the classification of these new techniques according to the mentioned European Directives and the German GenTG thereby considering the assessment carried out to date at the European level. The assessment carried out here relates to the application of the new techniques in plants and plant cells.

II. Legal basis

Directive 2001/18/EC, Directive 2009/41/EC and the GenTG each contain the definition of a GMO as well as non-exhaustive, indicative lists of techniques of genetic modification of organisms and of techniques which do not give rise to GMOs within the meaning of the legislation. Using these definitions and lists, the ZKBS carried out an expert analysis to determine which of the new techniques lead to genetic modification of the resulting organism.

According to Article 2, No. 2 of Directive 2001/18/EC a GMO is

"[…] 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; within the terms of this definition:

(a) genetic modification occurs at least through the use of the techniques listed in Annex I A, Part 1;

(b) the techniques listed in Annex I A, Part 2, are not considered to result in genetic modification; […]"

and according to Annex I A, Part 1 the following applies:

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

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

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

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

and according to Annex I A, Part 2 the following applies:

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

(1) in vitro fertilisation,

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

(3) polyploidy induction."

According to Article 3, No. 1 the following techniques constitute an exception in this regard; they do not result in any genetic modification of organisms:

"(1)This Directive shall not apply to organisms obtained through the techniques of genetic modification listed in Annex I B."

and according to Annex I B the following applies:

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

(1) mutagenesis,

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

According to Article 2(b) of Directive 2009/41/EC a GMO is

„ […] a micro-organism in which the genetic material has been altered in a way that does not occur naturally by mating and/or natural recombination; within the terms of this definition:

(i) genetic modification occurs at least through the use of the techniques listed in Annex I, Part A;

(ii) the techniques listed in Annex I, Part B, are not considered to result in genetic modification; […]"

and according to Annex I, Part A the following applies:

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

1. *Recombinant nucleic acid techniques involving the formation of new combinations of genetic material by the insertion of nucleic acid molecules produced by whatever means outside an organism, into any virus, bacterial plasmid or other vector system and their incorporation into a host organism in which they do not naturally occur but in which they are capable of continued propagation.*
2. *Techniques involving the direct introduction into a micro-organism of heritable material prepared outside the micro-organism, including micro-injection, macro-injection and micro-encapsulation.*
3. *Cell fusion or hybridisation techniques where live cells with new combinations of heritable genetic material are formed through the fusion of two or more cells by means of methods that do not occur naturally."*

and according to Annex I, Part B the following applies:

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

1. in vitro fertilisation;

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

3. polyploidy induction."

Thereby according to Article 3, No. 1 the following methods constitute an exception; they do not result in any genetic modification of organisms:

„1. Without prejudice to Article 4(1), this Directive shall not apply:

(a) where genetic modification is obtained through the use of the techniques/methods listed in Annex II, Part A; […]"

and according to Annex II, Part A the following applies:

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

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

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

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

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

According to § 3(3) GenTG

"a genetically modified organism is an organism, with the exception of human beings, whose genetic material has been altered in a way that does not occur naturally by mating or natural recombination; a genetically modified organism is also an organism which was generated by mating or natural recombination between genetically modified organisms or with one or more genetically modified organisms or by other methods of reproduction of a genetically modified organism, provided that the genetic material of the organism displays characteristics which arise from genetic engineering activities",

and according to § 3(3a) GenTG

"Techniques for the modification of genetic material within this meaning [...] are, in particular,

a) recombinant nucleic acid techniques involving the formation of new combinations of genetic material by the insertion of nucleic acid molecules which were generated outside the organism into any virus, viroid, bacterial plasmid or other vector system into a host organism in which they do not naturally occur,

b) techniques involving the direct introduction into an organism of heritable genetic material which was generated outside the organism and which does not occur naturally in the organism, including micro-injection, macro-injection and micro-encapsulation,

c) cell fusion or hybridisation techniques where live cells with new combinations of heritable genetic material are formed through the fusion of two or more cells by means of methods which do not occur naturally [...],"

and according to § 3(3b) GenTG the following are not regarded as techniques of modification of genetic material:

"[...]

a) in-vitro fertilisation,

b) natural processes such as conjugation, transduction, transformation,

c) polyploidy induction techniques, provided they do not involve the use of genetically modified organisms or the application of recombinant nucleic acid molecules generated as defined under Nos. 3 and 3a. Furthermore, the following are not considered techniques of modification of genetic material:

a) mutagenesis and

b) cell fusion (including protoplast fusion) of plant cells of organisms which can exchange genetic material through traditional breeding methods, on the condition that no genetically modified organisms are used as recipients or donors,"

and according to § 3(3c) GenTG:

„provided that they do not involve any release into the environment or placing on the market and provided that genetically modified organisms are not used as recipients or donors, the following are also not regarded as techniques of modification of genetic material:

a) cell fusion (including protoplast fusion) of prokaryotic species that exchange genetic material by known physiological processes,

b) cell fusion (including protoplast fusion) of cells of any eukaryotic species, including the production of hybridomas and fusions of plant cells,

c) self-cloning of non-pathogenic, naturally occurring organisms consisting in

aa) the removal of nucleic acid sequences from the cells of an organism,

bb) the re-insertion of all or part of the nucleic acid sequence (or of a synthetic equivalent) into cells of the same species or into cells of phylogenetically closely related species, which can exchange genetic material by natural physiological processes and

cc) with or without prior enzymic or mechanical treatment.

Self-cloning may include the use of recombinant vectors if they have a long history of safe use in the organism in question."

There are differences in the texts of the Directives and the GenTG cited here. The European Directives 2001/18/EC and 2009/41/EC state in Annex I A, Part 1, No. 1 and Annex I, Part A(1), respectively, that the recombinant nucleic acid which is inserted into a recipient organism must be "*capable of continued propagation*" in order to meet the criteria for a technique of genetic modification. In the German GenTG this addition was removed. This condition was justified in the Second Act to Amend the GenTG as follows:

„[...] The Gentechnikgesetz must also cover organisms which have been genetically modified through the use of replication-defective viruses (e.g. adeno- or retroviruses). These viruses are no longer capable of replication (reproduction) in the host organism; however, the genetic modification is replicated (reproduced) together with the host organism. In order to avoid such misinterpretation the passage from the EU Directive should not be adopted."

This justification is not scientifically correct. If the named replication-defective viruses integrate into chromosomes of the host and are thus permanently replicated through the host, then according to the GenTG the host is clearly a GMO (§ 3(3a)(b) GenTG). A "misinterpretation" is not possible here. Therefore, according to the assessment of the ZKBS, it is not necessary to deviate from the European Directives.

III. Further principles for the assessment of the new techniques

The German GenTG and the European Directives 2001/18/EC and 2009/41/EC define recombinant nucleic acid as newly combined genetic material. The ZKBS concurs with the opinion of the NTWG that a segment of DNA must comprise at least 20 nucleotide pairs (NPs) in order to give rise to a recombinant nucleic acid. Statistically, a specific sequence of 20 NPs with a random distribution of the NPs occurs once in 4^{20} NP (1.1×10^{12} NP). Hence, any specific sequence of less than 20 NPs is to be expected in large genomes such as

maize (the haploid genome has 2.5 x 10^9 NP) with a certain degree of probability. A deliberate alteration of less than 20 NPs cannot be distinguished with sufficient certainty from an incidental occurrence of this sequence. Specific sequences of less than 20 NPs can be detected, however they are not suitable for determining the origin of these sequences. They cannot be differentiated from genetic modifications arising from conventional mutagenesis or natural mutation (incidental occurrence) (Cao et al., 2011)[1]. A mutation that is induced by mutagenesis techniques does not constitute a genetic modification according to point (a) of the second sentence of § 3(3b) GenTG (mutagenesis).

Some techniques involve the generation of a GMO in an intermediate step (intermediate organism), from which progeny that no longer carry the genetic modification are subsequently selected. Two types of intermediate GMOs are considered. One type has the transferred nucleic acid chromosomally integrated (type A intermediate organism; as e.g. in reverse breeding (IV.7), cisgenesis (IV.3) and grafting (IV.4). The integrated genetic material can be removed in a further step, e.g. by crossing and segregation or by other methods. In the other type (type B intermediate organism) the transferred nucleic acid is neither integrated into the chromosome nor is it capable of autonomous replication so that it is not passed on to the offspring. Its presence in the organism is temporally limited (as e.g. in the case in the zinc finger nuclease technique (IV.2) and RNA-dependent DNA methylation (IV.6)) and it is subsequently lost. The ZKBS endorses the assessment of the NTWG that, from a scientific viewpoint, the progeny of GMOs which demonstrably no longer contain any genetically modified nucleic acid are not GMOs. For that reason, when considering the application of a technique, the ZKBS, as appropriate, differentiates between the parent organism, the intermediate organism, which is a GMO, and the resulting organism, which is not a GMO.

IV. New Techniques

IV.1 Oligonucleotide-directed mutagenesis (ODM)

Technique

Oligonucleotide-directed mutagenesis involves the transfer of oligonucleotides of approximately 20 to 200 nucleotides in length into a cell in order to produce a site-specific mutation at a certain sequence. These mutations can consist of exchanges of one or a few NPs, short deletions or short insertions of the cell's own DNA. The technique is based on the sequence-specific interaction of the oligonucleotide with its target sequence in the cell genome (*gene targeting*). Different types of oligonucleotides are used (Laible et al., 2006; Simon et al., 2008; Storici, 2008). These include single-stranded DNA with e.g. one or a few nucleotides that differ from those of the target sequence, chimeric oligonucleotides with fragments of RNA and DNA, oligonucleotides that form a triple helix with the target sequence by Hoogsteen hydrogen bonding (triplex-forming oligonucleotides; TFO) and RNA oligonucleotides with one or a few nucleotides divergent from the target sequence. Some of the oligonucleotides, so-called locked nucleic acids (LNA), containing modified nucleobases and/or modified ribose are also used in order to increase binding to the target sequence. Suitable for triple-helix formation are also nucleobases linked by peptide bonds (so-called peptide nucleic acids; PNA).

[1] In the sequenced genomes of 80 isolates of thale cress (*Arabidopsis thaliana*) from different geographic regions, 810,467 insertions and deletions of one to 20 NPs were found.

The cellular mechanisms, all of which result in mutation, are not completely understood. The DNA oligonucleotides might trigger mutations according to the principle of gene conversion. The triple-helix regions, which are formed by TFOs, represent target points for cellular DNA repair enzymes so that a double-strand break (DSB) can arise. This can lead to repair by non-homologous joining of the DNA ends (non-homolgous end joining; NHEJ) resulting in a point mutation or a short deletion or insertion (of the cell's own DNA). RNA oligonucleotides possibly serve as a matrix for the DNA repair, thereby triggering the mutation.

Gene-specific mutagenesis with oligonucleotides has already been applied successfully in crop plants (oilseed rape, maize, tobacco, rice, wheat) e.g. to generate herbicide tolerance. Other names for the technique include oligonucleotide-directed gene repair or oligonucleotide-targeted gene editing.

Conclusion and assessment

- The oligonucleotides which are introduced into cells do not represent new combinations of genetic material because their sequence is determined by the target sequence (Watson-Crick base pairing or Hoogsteen base pairing), in some cases with a deviation of one or a few nucleotides. The inserted oligonucleotides are not recombinant nucleic acids according to § 3(3a)(a) GenTG. The same assessment results according to Annex I A, Part 1, No. 1 of Directive 2001/18/EC and Annex I, Part A, No. 1 of Directive 2009/41/EC.
- The oligonucleotides including the chemically modified nucleic acids and derivatives do not constitute genetic material or heritable material according to § 3(3a)(b) GenTG. The same assessment results according to Annex I A, Part 1, No. 2 of Directive 2001/18/EC and Annex I, Part A, No. 2 of Directive 2009/41/EC.
- The oligonucleotides act as mutagens inducing mutations of one or a few NPs as can also occur spontaneously or following the application of mutagens, and can therefore not be differentiated from spontaneous mutations or from mutations induced by mutagenesis. Genetic variants produced by mutagens are not GMOs according to point (a) of the second sentence of § 3(3b) GenTG (mutagenesis). The same assessment results according to Annex I B of Directive 2001/18/EC and Annex II Part A, No. 1 of Directive 2009/41/EC.

Organisms which have been generated using the ODM technique are not GMOs. This assessment concurs with the assessment carried out by the NTWG.

IV.2 Zinc Finger Nuclease Technique

Technique

This technique allows the targeted induction of mutations in a genome, including insertion of large DNA segments and formation of deletions. Zinc finger nucleases (ZFNs) are chimeric proteins composed of two functional domains. The zinc finger domain binds to a specific nucleotide sequence (target sequence) in double-stranded DNA. The second domain harbours the endonuclease activity of the restriction enzyme *Fok*I. In this way, ZFNs produce a DNA single-strand break next to their target sequence; if two ZFN enzymes bind in opposite directions they can produce a DSB. Depending on the applied variant of the technique (ZFN1, ZFN2, ZFN3) different genetic modifications occur at the site of this DSB.

ZFNs can be inserted into an organism in a number of different ways. The genes for ZFNs can occur on a transiently present recombinant DNA in the organism. This results in a type B intermediate organism. Alternatively, ZFN m-RNAs or ZFN proteins can be inserted directly.

Other DSB-producing endonucleases with target sequences, e.g. meganucleases (Grizot et al., 2010) and *transcription activator-like endonucleases* (TALEN) (Christian et al., 2010) are used in a similar way to ZFNs.

There are three ZFN techniques:

In the first ZFN technique (ZFN1) the produced DSB can be repaired by the cell's own NHEJ DNA repair mechanism. This results in random mutations which affect one or a few NPs, or in short insertions (of the cell's own DNA) or short deletions. If two ZFN pairs are applied to target sequences that are far apart, a large deletion can be produced that is bordered by the target sequences.

In the second ZFN technique (ZFN2) DNA is transferred into the organism along with ZFNs. The transferred DNA can comprise several thousand NPs and is homologous to the flanks of the DSB produced by the ZFNs. At the DSB location the DNA differs by a mutation from the endogenous sequence. During DSB repair by homologous recombination with the transferred DNA, the nucleotide exchange is integrated into the genome.

In the third ZFN technique (ZFN3) DNA is transferred into the organism along with ZFN which allows integration of several thousand NP long DNA segments at the target sequence (directed integration). The transferred DNA segment is flanked by sections homologous to the DNA around the target sequence. The DNA segment is integrated into the chromosome during DSB repair.

Conclusion and assessment

Introduction of ZFNs into cells

- ZFNs can be produced in the organism by transiently present recombinant DNA with the genes for ZFNs (type B intermediate organism).

  According to § 3(3a)(a) GenTG the intermediate organism is a GMO. According to Annex I A, Part 1, No. 1 of Directive 2001/18/EC and Annex I, Part A, No. 1 of Directive 2009/41/EC the intermediate organism is not a GMO.

- If ZFNs are supplied to the cell through the transfer of isolated mRNA or isolated proteins, heritable genetic material is not introduced into the cell. For cells, RNA is not genetic material.

  Cells with ZFNs generated in this way are not GMOs according to § 3(3a)(b) GenTG. The same assessment results according to Annex I A, Part 1, No. 2 of Directive 2001/18/EC and Annex I, Part A, No. 2 of Directive 2009/41/EC.

Resulting organisms

- In the case of ZFN1 the progeny of the intermediate organism (the resulting organisms) only possess mutations that have arisen as a result of the natural process of NHEJ. These types of mutations can likewise result from natural processes or conventional breeding techniques (mutagenesis); the same applies to the production of large deletions.

  The resulting organism is not a GMO according to point (a) of the second sentence of § 3(3b) GenTG (mutagenesis). The same assessment results according to Annex I B, No. 1 of Directive 2001/18/EC and Annex II, Part A, No. 1 of Directive 2009/41/EC.

- Compared to the endogenous DNA, the DNA added by ZFN2 has a mutation of only one or a few NPs or a small insertion or deletion (less than 20 NPs). According to the definition given under "III. Further principles for the assessment of new techniques" this is not a recombinant nucleic acid. Hence the resulting organism does not carry a genetic modification covered by GMO regulations.

  The resulting organism is not a GMO according to § 3(3a)(a) GenTG. The same assessment results according to Annex I A, Part 1, No. 1 of Directive 2001/18/EC and Annex I, Part A, No. 1 of Directive 2009/41/EC.

- The DNA added by ZFN3 is a recombinant nucleic acid. If this recombinant DNA is chromosomally integrated, the resulting organism will be a carrier of a genetic modification.

  The resulting organism is a GMO according to § 3(3) GenTG. The same assessment results according to Article 2 of Directive 2001/18/EC and Article 2 of Directive 2009/41/EC.

With the exception of the evaluation of Type B intermediate organisms the assessment carried out here concurs with the assessment carried out by the NTWG.

IV.3 Cisgenesis and intragenesis

Technique

Cisgenesis involves taking a complete gene including its natural promoter and terminator regions and introns from an organism of the same species or a cross-compatible species (then referred to as the cisgene) and inserting it into the genome of a recipient organism (Jacobsen and Schouten, 2007; Rommens, 2007). Cisgenes frequently exhibit sequence identity or similarity with the nucleotide sequence of the recipient organism. The cisgenes are transferred using methods which are also applied in genetic engineering. To detect transformed organisms marker genes are transferred along with the cisgene (type A intermediate organism). These marker genes can be removed by further methods. At most, short nucleotide sequences of up to 10 NPs (e.g. recognition sequences of restriction endonucleases) remain in the resulting organism. If the transformation occurs by T-DNA transfer from *Agrobacterium* (Pitzksche and Hirt, 2010), T-DNA border sequences may remain in the recipient organism. The site at which the cisgene integrates in the genome is, e.g. in the case of T-DNA transfer, random, so that a gene localised there can be affected. By using targeted integration (e.g. when applying ZFN technique) an effect on other open reading frames (ORF) can be avoided. Cisgenes lead directly to genetic modifications which can also be achieved by crossing, but this takes longer and involves further genetic modification of the recipient organism.

In the case of intragenesis the transferred DNA also derives from the same species or a cross-compatible species. But the DNA (the intragene) is e.g. a combination of different gene sequences, a gene with promoter and terminator regions other than the native ones, or it can be the arrangement of the gene in sense or antisense orientation. New goals which cannot be realised with conventional breeding techniques can thus be achieved.

Conclusion and assessment

Cisgenesis

- As long as border sequences and/or marker genes are present, the resulting organism is considered a GMO according to § 3(3) GenTG. The same assessment results according to Article 2 of Directive 2001/18/EC and Article 2 of Directive 2009/41/EC.
- If no border sequences are present and the marker genes have been removed, cisgenesis is equivalent to self-cloning, since the resulting organism no longer exhibits any foreign nucleic acids. The resulting organism is not a GMO according to § 3(3c)(c) GenTG and Annex II, Part A, No. 4 of Directive 2009/41/EC provided it is exclusively intended for contained use. According to Article 2 of Directive 2001/18/EC the resulting organism is to be treated as a GMO if intended for use in deliberate releases into the environment and/or placing on the market.

Intragenesis

- Intragenesis involves the use of a DNA construct which is not present in either the recipient or the donor organism, but which consists of nucleotide sequences from the donor. This DNA construct is generated using nucleic acid recombination techniques.

  According to § 3(3a)(a) GenTG the resulting organism is a GMO. The same assessment results according to Annex I A, Part 1, No. 1 of Directive 2001/18/EC and Annex I, Part A, No. 1 of Directive 2009/41/EC.

The assessments carried out here concur with the assessments of the NTWG. Deviating from the final report of the NTWG, the ZKBS finds that cisgenesis is equivalent to self-cloning only if border sequences and marker genes are not present in the cisgene.

IV.4 Grafting

Technique

Grafting is a classic technique used to improve trees and herbaceous plants whereby a shoot or scion is attached to a rootstock. Through the formation of a callus the two parts grow together and the vascular systems join up. In the area around the joint organelles may be exchanged and mixed tissue may form (Stegemann and Bock, 2009). It has been shown that the chromosomal genes do not make their way from the root to the shoot or vice versa (Stegemann and Bock, 2009). Genetically modified plants (GM plants) can be used as the rootstock or shoot for grafting. The GM part of the chimeric plant can influence the non-GM part. Therefore, a suitable GM rootstock can e.g. increase the shoot growth and fructification of grafted plants on saline soils (Ghanem et al., 2011). The transfer of short RNA molecules from the root to the shoot, which e.g. lead to regulation by RNA interference and RNA-dependent DNA methylation, has been observed (Shaharuddin et al., 2006; Molnar et al., 2010).

Conclusion and assessment

- When a GM plant is used as the rootstock or shoot the chimeric organism has a GM part which may be reproductive: the shoot via flower and seed formation and the rootstock via root suckers (with flower and seed formation). Chimeric plants with either GM rootstocks or GM shoots are GMOs according to § 3(3) GenTG. The same assessment results according to Article 2 of Directive 2001/18/EC and Article 2 of Directive 2009/41/EC.
- The non-GM part of the chimeric plant does not undergo any genetic modification of its heritable genetic material. If a non-GM shoot is grafted onto a GM rootstock, the shoot will produce seeds and fruit which do not carry any genetic modification in their heritable genetic material and accordingly their offspring will not exhibit any genetic modification either.

  These progeny are not GMOs according to § 3(3) GenTG. The same assessment results according to Article 2 of Directive 2001/18/EC and Article 2 of Directive 2009/41/EC.
- If a GM shoot is grafted onto a non-GM rootstock the plant, fruit and seeds (i.e. the progeny) are carriers of a genetic modification.

  These plants and their progeny are GMOs according to § 3(3) GenTG. The same assessment results according to Article 2 of Directive 2001/18/EC and Article 2 of Directive 2009/41/EC.

The present assessment of the grafting technique and of the resulting seeds and fruit concurs with the assessment carried out by the NTWG.

IV.5 Agro-infiltration

Technique

With this technique plant tissue is treated (infiltrated) with a suspension of *Agrobacterium* spp. e.g. by rubbing, injection or by applying vacuum pressure. Agrobacteria have the capability to transfer the T-DNA containing in the transgene to plant cells (Pitzksche and Hirt, 2010).

Three types of agro-infiltration can be distinguished.

Agro-infiltration *sensu stricto*: Somatic tissue, i.e. tissue which does not contain or give rise to gametes, is infiltrated with agrobacteria. A DNA segment which is incapable of replication and which contains the transgene is introduced into the cells. The transgene is expressed in the infiltrated tissue.

Agro-inoculation/agro-infection: Somatic tissue is infiltrated with agrobacteria whose T-DNA harbours a viral vector on which the transgene is present. In this way increased expression of the transgene is achieved which can also take place in neighbouring or distant tissue.

Both agro-infiltration *sensu stricto* and agro-inoculation/agro-infection are used e.g. to produce certain proteins in plants, to temporarily turn off genes (gene silencing), or to examine promotor effects. Both techniques can lead to stable integration of the recombinant T-DNA in the genome of the somatic cells.

Floral dip: With this technique the gamete-forming organs (flowers) are infiltrated by immersion in an *Agrobacterium* suspension so that T-DNA also makes its way into gametes and embryos. The transferred T-DNA can integrate stably into the recipient genome and be chromosomally present in the resulting plant. This technique is widely used to produce stably transformed flowering plants.

Conclusion and assessment

Agro-infiltration *sensu stricto* and agro-inoculation/agro-infection

- Both of these techniques involve the transfer of T-DNA to somatic recipient cells. The T-DNA and its resulting products are present locally (non-replicating construct) or systemically (viral vector). The infiltrated plant is not a GMO according to § 3(3) GenTG. However, the infiltrated plant contains GMOs (agrobacteria or, in some cases, viruses). In the case of seed-transmissible viruses, the seed can contain GMOs. The same assessment results according to Article 2 of Directive 2001/18/EC and Article 2 of Directive 2009/41/EC.
- The T-DNA transferred by these two techniques can integrate into the genome of the somatic cells of the recipient organism. Multi-cellular organisms in which recombinant DNA is chromosomally present in parts of the somatic tissues but not in the reproductive tissues or germline cells are not classified as GMOs by the ZKBS because the recombinant DNA cannot be passed on to their progeny (ZKBS, 2011).

Floral dip

- When applying the floral dip method gametes and embryos are also infiltrated, so that T-DNA can integrate stably into the genome of gametes and of reproductive cells. T-DNA can thus be passed on to subsequent generations.

  The resulting organisms are GMOs according to § 3(3) GenTG. The same assessment results according to Article 2 of Directive 2001/18/EC and Article 2 of Directive 2009/41/EC.

The assessments carried out here on plants which result from agro-infiltration *sensu stricto*, agro-inoculation/agro-infection and floral dip techniques are in agreement with the assessments of the NTWG. However, the ZKBS does not consider infiltrated plants which may contain a number of transformed somatic cells, as GMOs; a definitive assessment of this point has not been carried out by the NTWG.

IV.6 RNA-directed DNA methylation (RdDM)

Technique

With the RdDM technique it is possible to turn off the expression of specific genes without altering the nucleotide sequence of the organism. The technique is based on the targeted methylation of the respective promoter which is thereby inactivated (Suzuki et al., 2005; Kanazawa et al., 2011). Targeted methylation is triggered by small interfering RNA (siRNA) or microRNA (miRNA) which exhibit sequence homology to the promoter. Several components of the cell's natural RNA-based regulation processes are involved in this process (Molnar et al., 2011). The DNA methylation pattern is maintained by special maintenance methyltransferases and is passed on to the progeny. In this way, promoter inactivation (epigenetic effect) can persist in the plant which is regenerated from the cell and can also be passed on through several generations, although eventually it will be lost again.

The necessary siRNA or miRNA can be provided in a number of ways, for example:

- The RNA can be introduced into the cell by transferring a recombinant DNA with the gene for the required RNA followed by integration of the DNA into a chromosome.
- The RNA can be introduced by transferring a recombinant DNA with a gene for the RNA which is only transiently present and expressed in the cell. This cell is a type B intermediate organism. Only the epigenetic modification that results from this procedure will persist in the progeny of this organism.
- Isolated RNA can be introduced directly into the cell.

Conclusion and assessment

- If recombinant DNA is stably integrated into the genome of the recipient organism for the generation of siRNA or miRNA, the organism is genetically modified.

  According to § 3(3a)(a) GenTG this organism is regarded as a GMO. The same assessment results according to Annex I A, Part 1, No. 1 of Directive 2001/18/EC and Annex I, Part A, No. 1 of Directive 2009/41/EC.

- If the recombinant DNA is present only transiently, it is present only in the intermediate organism but not in its progeny.

  The intermediate organism is a GMO according to § 3(3a)(a) GenTG. According to Annex I A, Part 1, No. 1 of Directive 2001/18/EC and according to Annex I, Part A, No. 1 of Directive 2009/41/EC the intermediate organism is not a GMO.

  Progeny of the intermediate organism which have been shown not to contain recombinant DNA and which only exhibit the epigenetic modification are not to be assessed as GMOs because they do not differ genetically from the parent organism (§ 3(3) GenTG and also Article 2 of Directive 2001/18/EC and Article 2 of Directive 2009/41/EC).

- If siRNA or miRNA is made available in the cell by the introduction of isolated RNA it does not result in the transfer of heritable genetic material into the cell. For cells, RNA is not genetic material.

According to § 3(3a)(b) GenTG this organism is not a GMO. The same assessment results according to Annex I A, Part 1, No. 2 of Directive 2001/18/EC and Annex I, Part A, No. 2 of Directive 2009/41/EC.

The present assessment of organisms produced with the RdDM technique concurs with the assessment of the NTWG.

IV.7 Reverse Breeding

Technique

With the reverse breeding technique significant amounts of a specific F1 hybrid can be produced with relative ease (Dirks et al., 2009). F1 hybrid seed is agronomically important for exploiting the heterosis effect. Over several steps, reverse breeding produces two parent plants from a selected hybrid with the desired traits, which when subsequently crossed give rise exclusively to unaltered hybrids (Wijnker et al., 2012). In the first step, cells of the F1 hybrid are transformed by stable chromosomal integration of an RNAi construct in order to block meiotic recombination by silencing the necessary genes (e.g. SPO11 or DCM1). From these transformed cells several transformed lines are regenerated which carry the RNAi construct either in different chromosomes or in different members of pairs of homologous chromosomes (resulting in type A intermediate organisms). Suppression of meiotic recombination can also be achieved by inserting transiently present siRNA- or miRNA-producing vectors (resulting in type B intermediate organisms). These plants produce (haploid) gametes with chromosomes which are not altered by crossing over. Conventional breeding methods can be used to induce genome doubling in gametes of the intermediate organism. This results in plants that possess two copies of the haploid genome (so-called “double haploids”; step 2). Finally, two of the “double haploids” are selected which have sets of chromosomes complementing each other to give the desired hybrid and which at the same time do not carry any RNAi construct (step 3; resulting organisms). These are the desired two homozygous parental lines for hybrid production and can be reproduced by selfing.

Conclusion and assessment

Step 1 of the technique

- If a recombinant DNA is stably integrated into the genome of the parent organism in order to suppress meiotic recombination, a type A intermediate organism with a heritable genetic modification is generated.

  The resulting organism is a GMO according to § 3(3) GenTG. The same assessment results according to Article 2 of Directive 2001/18/EC and Article 2 of Directive 2009/41/EC.

- If recombinant nucleic acids which are only transiently present are used to suppress the meiotic recombination, a type B intermediate organism is generated.

  The intermediate organism is a GMO according to § 3(3a)(a) GenTG. According to Annex I A, Part 1, No. 1 of Directive 2001/18/EC and according to Annex I, Part A, No. 1 of Directive 2009/41/EC the intermediate organism is not a GMO.

Step 2 of the technique

- The generation of double haploids is based on standard techniques used in conventional breeding; these techniques do not involve the use of methods of genetic engineering.

  The resulting organisms are not GMOs according to § 3(3a) GenTG. The same assessment results according to Annex I A, Part 1 of Directive 2001/18/EC and Annex

I, Part A of Directive 2009/41/EC. If this technology is applied to GMOs they shall remain GMOs according to § 3(3) GenTG.

Step 3 of the technique

- The organisms resulting from reverse breeding have been shown to be free of the recombinant nucleic acids used in the previous steps.

  The resulting organisms are not GMOs according to § 3(3) GenTG. The same assessment results according to Article 2 of Directive 2001/18/EC and Article 2 of Directive 2009/41/EC.

The present assessment of the reverse breeding technique and organisms resulting from this technique is in agreement with the assessment carried out by the NTWG.

V. References

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