CRITERIA FOR GROUPING NEW PLANT BREEDING TECHNIQUES

Regulators and risk assessors in different countries started to evaluate plants derived through new plant breeding techniques (NPBTs) technique-by-technique or event-by-event[1]. In order to achieve a more consistent and effective evaluation process we suggest that a more structured approach can be developed. As a first step in this process we present a list of criteria which can be regarded as relevant for evaluating NPBTs in a structured way.

The following analysis is based on information compiled in the framework of a study carried out by the JRC in the year 2010[2] and covers the following new plant breeding techniques:

1. Zinc finger nuclease (ZFN) technology (ZFN-1, ZFN-2 and ZFN-3)
2. Oligonucleotide directed mutagenesis (ODM)
3. Cisgenesis and intragenesis
4. RNA-dependent DNA methylation (RdDM)
5. Grafting on GM rootstock
6. Reverse breeding
7. Agro-infiltration (agro-infiltration "sensu stricto", agro-inoculation and floral dip)

1 Lusser, M., Rodríguez-Cerezo, E., 2012. Comparative regulatory approaches for new plant breeding techniques. Workshop proceedings. European Commission. JRC Technical Report EUR 25237 EN.

2 Lusser, M., Parisi, C., Plan, D., Rodríguez-Cerezo, E., 2011. New plant breeding techniques: State-of-the-art and prospects for commercial development. JRC Technical Report EUR 24760 EN.

DEFINITIONS:

1. **Zinc finger nuclease (ZFN) technology (ZFN-1, ZFN-2 and ZFN-3)**

Zinc finger nucleases are proteins combining a DNA cleavage domain (usually the *Fok*I endonuclease) and a DNA binding domain (with several Cys2His2 zinc fingers) that is custom-designed to bind to a specific site of the genome. Two ZFN genes (ZFNs act in pairs) are usually delivered into the cell in an expression plasmid. Depending on the ZFN technique variant (ZFN-1 to -3), a short repair template or a template containing a long stretch of DNA can be delivered additionally. The two ZFNs expressed by the inserted gene bind to adjacent DNA sequences enabling the *Fok1* endonuclease to form a dimer and create a double strand break (DSB) at a specific site in the DNA. The double strand break stimulates the cell's natural mechanisms to repair the break through non homologous end joining (NHEJ) or homologous recombination (HR), thus facilitating site-specific mutagenesis.

In the case of **ZFN-1,** no repair template is provided. NHEJ of the DSB leads to site-specific random mutations (deletions, insertions or changes of one or a few base pairs). In the case of short insertions the inserted material is from the organism's own genome.

For **ZFN-2** the expression plasmid contains in addition to the ZFN genes a short repair template which is homologous to the target area with the exception of the base pair(s) to be changed. Repair through HR of the DSB and the copying of the repair template lead to a site-specific, pre-determined mutation (short deletions, insertions or substitutions with one or more specific base pairs).

In the case of **ZFN-3,** DNA fragments of up to several kilo base pairs whose ends are homologous to the DNA sequences flanking the cleavage site are introduced along with the ZFN genes. As a result, the DNA fragment is inserted into the plant genome by HR in a site-specific manner.

2. **Oligonucleotide-directed mutagenesis (ODM)**

ODM deploys chemically synthesised oligonucleotides (e.g. single-stranded DNA oligonucleotides or chimeric oligonucleotides including DNA and RNA bases) which share homology with the target sequence of the plant genome with the exception of one or more base pairs. The oligonucleotides target the homologous sequence in the genome of the host and induce site-specific nucleotide substitutions, insertions or deletions through the natural repair mechanism of the cell.

3. Cisgenesis and intragenesis:

Cisgenesis leads to a genetic modification of a recipient organism through insertion of a gene from a crossable organism (same species or closely related species). This gene which includes its introns, is flanked by its native promoter and terminator and is inserted in normal sense orientation. In the case of intragenesis, the inserted DNA can be a new combination of DNA fragments from the species itself or from cross-compatible species which can be inserted in sense or anti-sense orientation.

4. RNA dependent DNA methylation (RdDM)

RdDM is achieved by insertion of genes encoding RNAs which are homologous to the promoter regions of the target gene leading to the production of a transgene. Transcription of the gene leads to double stranded RNA which after cutting by specific enzymes into sRNAs (small RNAs) induces methylation of the promoter region of the target gene. This leads to gene silencing through inhibition of the transcription of the target gene (transcriptional gene silencing, TGS). The change of the methylation pattern will be inherited by the following generation even in the absence of the inserted transgene. Although the methylation status can be inherited over a number of generations, the epigenetic effect will eventually fade out.

5. Grafting on a genetically modified rootstock

Grafting is a horticultural technique whereby the above ground vegetative component of one plant (known as scion) is attached to the rootstock of another plant to produce a chimeric organism. The technique combines the desired properties of a rootstock with those of the scion. The plant for the rootstock can be transformed by transgenesis, cisgenesis or other techniques.

6. Reverse breeding

Reverse breeding comprises several consecutive steps. First a transgene encoding RNA interference (RNAi) sequences is delivered to explants and a transgenic plant is regenerated in tissue culture. Silencing of genes such as *dmc1* and *spo11* leads to suppression of meiotic recombination and haploid microspores (immature pollen grains) are produced from flowers of the transgenic plant. The microspores develop into homozygous diploid plants (doubled haploid technology) under suitable cell culture conditions. In a final step plants that do not contain the transgene and whose hybridization reconstitutes the elite heterozygous line are selected.

7. Agro-infiltration:

Depending on the tissues and the type of constructs infiltrated, three variants of agro-infiltration can be distinguished.

In the case of **agro-infiltration "sensu-stricto"** the technology is applied to intact plants or detached plant parts. Non-germline tissue, mostly leaves are infiltrated with a liquid suspension of *Agrobacterium sp.* containing the gene of interest to obtain localized expression in the infiltrated area.

For **agro-infiltration** non-germline tissue, mostly leaves are infiltrated with a liquid suspension of *Agrobacterium sp.* containing the foreign gene in a full-length virus vector to facilitate systemic spreading and expression of the target gene in the entire plant.

In the case of **floral dip**, germline tissue (typically flowers) is immersed into a suspension of *Agrobacterium* carrying a DNA construct in order to obtain transformation of some embryos that can be selected at the germination stage. The aim is to obtain stably transformed plants.

CRITERIA:

RATIONAL FOR APPLICATION OF THE TECHNIQUE

1 Which technique (NPBT) is used, where if fits in the breeding process and the effect achieved

It can take up to 15 years to develop a new commercial plant variety through conventional breeding depending on crop and trait (up to 50 years in the case of fruit trees). Plant breeding starts with the creation of new genetic variation (if not occurring naturally), followed by selection which requires several years of field assessment. Following such an evaluation, the new variety can be multiplied and released.

The majority of techniques discussed here enable the creation of new genetic variation which is achieved by modification of existing genes, the insertion of a new gene, the replacement of a gene with another allele or other approaches (e.g. substitution of a whole chromosome of the genome, see reverse breeding below).

Other techniques such as agro-infiltration are used for selecting suitable plants coming from one breeding cycle for the use in the next cycle. Through techniques such as accelerated breeding following early flowering it is possible to shorten the duration of a breeding cycle.

Other NPBTs which are used following the development of the desired genotype include a) reversed breeding for the production of homozygous parental lines for the production of F1 hybrids and b) grafting which is a horticultural technique combining the scion of one plant with the rootstock of another plant thus combining favourable features of both plants.

TECHNIQUE	Creation of new genetic variation	Breeding cycles/ selection	Breeding steps following the development of the wished genotype e.g. multiplication or horticultural techniques
ZFN-1	Site-specific, random mutations (deletions, exchange or insertion of one or more base pairs) leading e.g. to gene-knock-out or more effective alleles.		
ZFN-2	Site-specific, pre-determined mutations (deletions, exchange or insertion of one or more base pairs) leading e.g. to gene-knock-out or more effective alleles.		
ZFN-3	New gene is inserted at target site or an existing one replaced with another resulting in new or improved traits.		
ODM	Site-specific, pre-determined mutations (deletions, exchange or insertion of one or more base pairs) leading e.g. to gene-knock-out or more effective alleles.		
Cisgenesis	Gene insertion leading to new or improved traits.		
Intragenesis	Gene insertion leading to new or improved traits. RNAi or anti sense can be also used (gene silencing).		
RdDM	Gene silencing achieved through methylation of promoter sequence of the target gene. The effect is heritable, but fades out after several generations. (a)	Potential use for breeding with transgenic inducer line such as reverse breeding or early flowering. (b)	
Grafting on GM rootstock	(Creation of new variation is relevant for the breeding of the GM rootstock.		Grafting is a horticultural technique producing a chimera of one plant

TECHNIQUE	Creation of new genetic variation	Breeding cycles/ selection	Breeding steps following the development of the wished genotype e.g. multiplication or horticultural techniques
	This can be achieved by transgenesis, cisgenesis or other suitable techniques.)		serving as rootstock and another serving as scion leading to e.g. improvement of the rootstock (c). Molecules such as proteins or RNA induced by the transgene can be transported to the scion and change gene expression leading e.g. to gene-silencing.
Reverse Breeding	Potential application for creation of new genetic variation, e.g. chromosome substitution lines (d).		Used for production of homozygous parental lines for the production of F1 hybrids in a much shorter timeframe than conventional breeding.
Agro-infiltration "sensu stricto"		Use for (i) selection for specific trait, (ii) for studying plant–pathogen interaction in leaves or the functionality of a gene construct or (iii) for plant molecular farming for the production of recombinant proteins in infiltrated plants. (e)	
Agro-infection		Use for (i) selection for specific trait or (ii) for studying plant-pathogen interaction in leaves or the functionality of a gene construct.	
Floral dip	New gene inserted resulting in a new trait. (f)		

(a) Therefore, it is questionable if this technique is useful for the introduction of new traits N.B: For plant variety registration stability of the trait is a requirement.

(b) In the case of these applications gene silencing is only required for a specific step in the breeding process and is no longer relevant in the commercialised crop.

(c) The rootstock can be improved e.g. through introduction of genes conferring resistance against soil born disease or better rooting ability.

(d) Reverse breeding can be used for the production of so-called chromosome substitution lines. These lines contain one or more chromosomes from one parent in the genetic background of another parent. This approach can be applied to improve parental lines.

(e) The infiltration can be carried out on both attached and detached plant parts. In the case of detached plant parts the experiments are often carried out in tissue culture conditions. In some cases, e.g. where there is a long latency period for the effect under study, it is necessary to work directly with whole plants and to rescue the plants with the interesting phenotype.

(f) Floral dip is an easy approach for achieving the insertion of a transgene as it avoids reconstitution of the plant from the explant through tissue culture. However, for practical reasons its application is restricted to plants with small flowers and it is mainly used with *Arabidopsis*.

METHOD OF APPLICATION

2 Molecules deployed

An important criterion for classification of NPBTs is the nature of the molecules deployed. It is especially important to specify DNA fragments which have been isolated from plants or other organisms and are used to develop the new plant variety as this may lead to intended or un-intended integrations of foreign DNA into the plant genome (see also sections 4 and 5) and eventually to transgenic plants.

Specific approaches in this context are cisgenesis and intragenesis which deploy only DNA from the species itself or cross compatible species (see also table below). In principle, all techniques using transgenes can be carried out also with cisgenes/intragenes, although due to the restricted conventional plant breeders' gene-pool, suitable genes may not always be available.

TECHNIQUE	DNA from outside the organism	Oligonucleotide, RNA	Protein
ZFN-1	Usually delivered as a plasmid vector carrying the gene encoding the ZFN.	Alternatively, delivery as mRNA encoding the ZFN is possible.	Possibility of delivering ZFNs directly as proteins has been demonstrated in mammalian cells.
ZFN-2	Usually delivered as a plasmid vector carrying the gene encoding the ZFN.	Alternatively, delivery as mRNA encoding the ZFN is possible.	Possibility of delivering ZFNs directly as proteins has been demonstrated in mammalian cells.
	Additionally a repair template is delivered which is homologous to the target sequence with the exception of one or more base pairs to be modified.		
ZFN-3	Usually delivered as a plasmid vector carrying the gene encoding the ZFN.	Alternatively, delivery as mRNA encoding ZFN is possible.	Possibility of delivering ZFNs directly as proteins has been demonstrated in mammalian cells.
	Additionally a repair template is delivered which consists of the gene of interest flanked by DNA		

TECHNIQUE	DNA from outside the organism	Oligonucleotide, RNA	Protein
	homologous with the DNA sequences flanking DSB site.		
ODM		Oligonucleotides (a) which share homology with the target sequence of the plant genome with the exception of one or a few base pairs.	
Cisgenesis	Vector carrying DNA sequences from the plant species itself or from cross-compatible plant species in their natural orientation. The inserted gene is unchanged and includes its own introns and regulatory sequences.		
Intragenesis	Vector carrying DNA fragments from the plant species itself or from cross-compatible plant species and not necessarily in sense or antisense orientation. The inserted gene consists of fragments of different genes from the species itself or from cross-compatible species.		
RdDM	Plasmid vector carrying a gene encoding ds RNA		
Grafting on GM rootstock	For breeding the plant which is used as rootstock: DNA vector carrying the transgene or cisgene is deployed		
Reverse Breeding	Plasmid vector carrying a gene encoding RNA interference (RNAi) sequences (b)		
Agro-infiltration "sensu stricto"	*Agrobacterium* construct containing gene of interest. (c)		
Agro-infection	*Agrobacterium* construct containing gene of interest in a full-length virus vector. (c)		
Floral dip	*Agrobacterium* construct containing gene of interest. (c)		

(a) E.g. single-stranded DNA oligonucleotides or chimeric oligonucleotides including DNA and RNA bases.

(b) For reverse breeding several consecutive steps are carried out, for the first step a plasmid vector carrying a gene encoding RNA interference (RNAi) sequences is delivered (see also sections 4).

(c) For the method of delivery see section 3.

3 Plant tissues or cells manipulated and method of delivery

The method of delivery and nature of the cells manipulated is usually determined by the aim of the application (see section 1) and the molecules deployed (see section 2). The choice of methodology will determine intended (see sections 1 and 6) and possible off target effects (see section 8) and therefore it will have implications for the classification under the GMO legislation and for the risk assessment.

Most NPBTs are applied to so-called explants which are plant fragments (e.g. leaf discs, root sections internodes or buds) or plant cells from tissue cultures (e.g. calli or cell suspensions) and the most frequently used methods are *Agrobacterium*-mediated transformation or biolistics. A pre-requisite for the use of explants in plant breeding is their ability to be regenerated in tissue culture into whole plants which are used in the further breeding process. In the case of agro-infiltration intact plant tissue is immersed into or infiltrated by *Agrobacterium* suspension thus avoiding the application of tissue culture.

TECHNIQUE	Explant	Intact plant, local application	Special case	Method of delivery
ZFN-1				*Agrobacterium*, biolistics, etc.
ZFN-2				*Agrobacterium*, biolistics, etc.
ZFN-3				*Agrobacterium*, biolistics, etc.
ODM				Electroporation (protoplasts), biolistics (plant fragments)
Cisgenesis and Intragenesis				*Agrobacterium*, biolistics, etc.
RdDM				*Agrobacterium*, biolistics, etc.
Grafting on GM rootstock	(When breeding plant for rootstock) (a)		Vegetative component of one plant (scion) is attached to the rootstock of another plant	
Reverse Breeding				*Agrobacterium*, biolistics, etc.

TECHNIQUE	Explant	Intact plant, local application	Special case	Method of delivery
Agro-infiltration "sensu stricto"		Applied to non-germline tissue		Infiltration by e.g. syringe with *Agrobacterium* suspension
Agro-infection		Applied to non-germline tissue		Infiltration by e.g. syringe with *Agrobacterium* suspension
Floral dip		Applied to germline-tissue		Immersion of flowers in *Agrobacterium* suspension

(a) Grafting is a horticultural technique whereby the above ground vegetative component of one plant (also known as the scion), is attached to the rootstock of another plant to produce a chimeric organism. Plants used for grafts can be transformed by transgenesis, cisgenesis or other techniques.

PROCESS

4 Process at molecular level following delivery of technology into the cell

Following the delivery of DNA fragments or other molecules into the cell (discussed in section 3), the next relevant step is the process at molecular level. Here stable changes in the genome and especially insertion of new genes are of importance for the final product and its classification vis-à-vis the GMO legislation. For most of the NPBTs the delivered DNA is stably inserted in the genome in a first step (even if plants carrying the inserted gene are segregated out during further breeding steps in many cases). ZFN genes are integrated into the plant as transgenes or ZFNs are transiently expressed from gene vectors. In the latter case the creation of transgenic plants are avoided (see also table below). Also for agro-infiltration "sensu-stricto" and agro-infection expression of the delivered genes without stable insertion is intended.

TECHNIQUE	Stable insertion of gene	Transient gene expression	Other
ZFN-1	ZFN genes are integrated into the plant as transgenes or ZFNs are transiently expressed from gene vectors (a)		ZFNs bind to DNA and cause DSBs. The DSB triggers a repair mechanism by non-homologous endjoining (NHEJ) leading to site specific mutations (b)
ZFN-2	ZFN genes are integrated into the plant as transgenes or ZFNs are transiently expressed from gene vectors (a)		ZFNs bind to DNA and cause DSBs. The DSB leads to a site-specific and defined mutations through homologous recombination (HR) and the copying of a short repair template. (b)
ZFN-3	ZFN genes are integrated into the plant as transgenes or ZFNs are transiently expressed from gene vectors (a)		ZFNs bind to DNA and cause DSBs. The gene of interest (included in a repair template) is inserted into the plant genome at the site of the DSB by HR. (b)
	Gene encoding trait of interest		
ODM			Oligonucleotides target the homologous sequence and create one or more mismatched base pairs corresponding to the non-complementary nucleotides in the oligonucleotide.
Cisgenesis	Genes from plant species itself or		

TECHNIQUE	Stable insertion of gene	Transient gene expression	Other
	cross-compatible species – unchanged and with its own regulatory elements and introns in natural sense. (c)		
Intragenesis	New combinations of DNA sequences from plant species itself or cross-compatible species – in sense or anti-sense orientation. (c)		
RdDM	DNA encoding dsRNA		Gene silencing through DNA methylation of promoter sequence of target gene. (d)
Grafting on GM rootstock	(Relevant for breeding of the rootstock.)		Recombinant proteins, hormones and non-coding RNA (e.g. siRNA) can be transported from the GM rootstock of a graft to the scion where they might induce effects such as gene-silencing or changes of protein concentration.
Reverse Breeding	Transgene encoding RNA interference (RNAi) sequences. (e)		(e)
Agro-infiltration "sensu stricto"	Integration of T-DNA fragments into the plant genome is possible. Absence of DNA fragments in final plant has to be proven.	The intended goal of the technique is the temporary expression of specific coding sequences without the integration of the introduced DNA in the plant genome.	
Agro-infection	The gene construct is inserted into a RNA virus genome. As spread via RNA molecules, no integration into the plant genome.	The intended goal of the technique is the temporary expression of specific coding sequences without the integration of the introduced DNA in the plant genome.	
Floral dip	Stable insertion of the gene – locally in germline tissue (e.g. flowers).		

(a) This applies when ZFNs are delivered into the cell as DNA. Alternatively, ZFNs can be delivered as mRNA or directly as proteins (see section 2).

(b) See also sections 1 and 2.

(c) In the case of *Agrobacterium*-mediated transformation insertion of so-called T-DNA borders have to be expected (see also section 7).

(d) See also section 1.

(e) For reverse breeding several consecutive steps are required. In the first step a transgene encoding RNA interference (RNAi) sequence is delivered to plant fragments and a transgenic plant is regenerated in tissue culture. Silencing of genes such as *dmc1* and *spo11* leads to suppression of meiotic recombination and haploid microspores (immature pollen grains) are produced from flowers of the transgenic plant. The microspores are developed to homozygous diploid plants (doubled haploid technology) under suitable cell culture conditions.

5 Intermediate (transgenic) plant produced - transgene segregated out during final breeding steps or not

Most of the NPBTs discussed here deploy DNA fragments which have been isolated from plants or other organisms and subsequently cloned (see also section 2). For some of the techniques the aim is stable integration of this DNA into the host genome in order to achieve a new or improved trait and its heritability in subsequent generations. In the case of so-called “breeding with transgenic inducer line" (which has also been referred to as "transgenic construct driven breeding") a transgene encoding an RNAi construct is present in the genome of an inducer line. The expression of the transgene leads to the inhibition of gene expression or the inhibition of a protein function. This can drive, for example, the suppression of meiotic recombination or early flowering. The inducer transgene is segregated out during further breeding and is therefore not present in the final product (the end product is a negative segregant).

TECHNIQUE	Inserted gene – segregated out	Inserted gene in final plant	Change in genome other than inserted gene in final plant
ZFN-1	Gene encoding ZFN (in the case of stable insertion (a)).	Absence of inserted ZFN gene in final plant has to be proven (b).	Once the ZFN gene is segregated out, the plant genome still carries mutations.
ZFN-2	Gene encoding ZFN (in the case of stable insertion (a)).	Absence of inserted ZFN gene in final plant has to be proven (b).	Once the ZFN gene is segregated out, the plant genome still carries mutations.
ZFN-3	Gene encoding ZFN (in the case of stable insertion (a)).	Absence of inserted ZFN gene in final plant has to be proven (b). Once the ZFN gene is segregated out, the plants still carry the gene of interest.	
ODM			Plant genome carries mutation. (c)
Cisgenesis and Intragenesis		(d)	
RdDM	DNA encoding dsRNA. (e)		Methylation of promoter sequence of the target gene. (e)

Grafting on GM rootstock			Grafting produces a chimeric plant consisting of a GM rootstock and a non-GM scion.
Reverse Breeding	Transgene encoding RNA interference (RNAi) sequences. (e)		
Agro-infiltration "sensu stricto"		(f)	
Agro-infection		(f)	
Floral dip		Progeny of the infiltrated plant is transgenic.	

(a) See section (4).

(b) This applies in the case of stable gene insertion and transient gene expression respectively (see section 4).

(c) The oligonucleotides are degraded in the cells.

(d) In the case of cisgenesis and intragenesis, the gene is stably integrated and inherited by the progeny.

(e) Application of breeding with transgenic inducer line.

(f) Absence of inserted gene and of *Agrobacterium* has to be proven.

PRODUCT

6 Nature of heritable change in the genome if any

NPBTs can lead to heritable changes in the genome of the final product but also to situations which leave the genome unchanged or where the change is only intended in an intermediate plant. Heritable changes in the genome are achieved by the NPBTs discussed here through mutations in existing genes, through the insertion of a new genes or allele replacement, or through other mechanisms such as the change of an entire chromosome (see reverse breeding, section 1).

TECHNIQUE	No intended change in genome	Stably inserted gene	Mutation Of on or a few base pairs	Other
ZFN-1			Mutations which are site specific but exchanged base pairs are not pre-defined.	
ZFN-2			Mutations which are site-specific and the introduced mutations are defined.	
ZFN-3		Transgene or cisgene/intragene of interest. (b)		
ODM			Mutations which are site-specific and the introduced mutations are defined.	
Cisgenesis		Gene from plant species itself or cross-compatible species – unchanged and with its own regulatory elements and introns in natural sense.		
Intragenesis		Combination of DNA sequences from plant species itself or cross-compatible species – in natural sense or anti-sense.		

TECHNIQUE	No intended change in genome	Stably inserted gene	Mutation Of on or a few base pairs	Other
RdDM	No change in sequence of base pairs intended.			Methylation of promoter sequence leads to epigenetic effect.
Grafting on GM rootstock	Crop (fruits) and progeny (seeds) are free from inserted transgenes.	Stable integration of a novel gene is achieved in the rootstock.		
Reverse Breeding	When used for the reproduction of parental lines for hybrids: Products do not differ from products achieved through conventional breeding.	For other applications e.g. for producing chromosome substitution lines: These lines contain one or more chromosomes from one parent in the genetic background of another parent.		
Agro-infiltration "sensu stricto"	After use of agro-infiltration for selection, progeny of the plant which is free from transgene or other changes will be used in the further breeding process.			
Agro-infection	After use of agro-infection for selection, progeny of the plant which is free from transgene or other changes will be used in the further breeding process.			
Floral dip		Stable insertion of the gene is intended and progeny of the infiltrated plant will be transgenic.		

7 Changes developed through the NPBT achievable through conventional breeding or occurring naturally?

When deciding on the classification of NPBTs under GM legislation, a consideration of whether or not the end product could also occur naturally or be achieved through conventional plant breeding is relevant. Many experts are of the opinion that plants derived through NPBTs and which could be also achieved through conventional breeding or through natural mutations should be exempted from the GM legislation. Under the EU legislation one criterion for the classification of recombinant nucleic acid technique as genetic modification is that the formation of new combinations of genetic material by the insertion of nucleic acid molecules does not naturally occur.

TECHNIQUE	Yes	No
ZFN-1	Mutations of one or a few base pairs resulting from ZFN-1 could also occur naturally or result from chemical treatment or irradiation.	But: Intermediate plant which is not achievable through conventional breeding and which might be considered transgenic. (a)
ZFN-2	Depending on mismatch in repair template either mutations of one or a few base pairs (which could occur naturally or through mutagenesis induced by irradiation or chemicals) or more complex directed changes possible (which are extremely unlikely to arise via natural means or via mutagenesis through chemicals and irradiation). In both cases: Intermediate plant which is not achievable through conventional breeding and which might be considered transgenic. (a)	
ZFN-3	For cisgenesis with 'narrow' definition (see below). But: Intermediate plant which is not achievable through conventional breeding and which might be considered transgenic. (a)	When gene from outside gene pool is used. Additionally: Intermediate plant which is not achievable through conventional breeding and which might be considered transgenic. (a)
ODM	Depending on mismatch in repair template either mutations of one or a few base pairs (which could occur naturally or through mutagenesis induced by irradiation or chemicals) or more extensive changes possible (which are extremely unlikely to arise via natural means or via mutagenesis through chemicals and irradiation).	
Cisgenesis	Cisgenesis deploys genes with its own regulatory elements and introns and in their natural sense from the plant species itself or from cross-compatible species. Similar crops, therefore, could also be developed by conventional breeding. However, in the case of *Agrobacterium*-mediated transformation, the insertion of short T-DNA border sequences has to be expected. Although sequences which are similar to these T-DNA border sequences are present in many plants, it has to be decided case by case, if plants derived by this approach can be regarded as also achievable by conventional breeding.	

TECHNIQUE	Yes	No
Intragenesis		Intragenesis allows the use of combinations of sequences from different genes from the plant species itself or from a cross-compatible plant, e.g. combining coding sequences from one gene with regulatory elements from other genes. Gene sequences can be inserted in their natural sense and also anti sense orientation. Such changes are extremely unlikely to occur during conventional breeding.
RdDM	Methylation of gene sequences leading to modification of gene expression is a natural process.	
Grafting on GM rootstock	Grafts with a rootstock transformed through cisgenesis (see above) might also be possible with conventional breeding.	Grafts with a rootstock transformed through transgenesis is not possible with conventional breeding.
Reverse Breeding	When used for the reproduction of parental lines for hybrids: Reverse breeding (like RdDM) uses a transgene only in the inducer line. Products from reverse breeding do not differ from products achieved through conventional breeding.	Other applications e.g. for producing chromosome substitution lines: These lines contain one or more chromosomes from one parent in the genetic background of another parent. It is very unlikely that such a plant will be achieved by conventional breeding.
Agro-infiltration "sensu stricto"	When used for selection, progeny of the plant which is free from transgene or other changes will be used in the further breeding process.	In case of use for molecular farming the infiltrated plant developed would not be achievable without biotechnology.
Agro-infection	After application of agro-infiltration for selection, progeny of the plant which is free from transgene or other changes will be used in the further breeding process.	
Floral dip	Cisgenic approach in principle possible. However, T-DNA border sequences have to be expected as *Agrobacterium* is used as carrier in this technique. (See discussion on cisgenesis above).	In the case of transgenic approach a transgene will be present in the final plant.

(a) In the case of stable gene insertion the plant is transgenic until the gene is segregated out. In such cases the plant becomes a negative segregant and thus may or may not fall under legislation depending on the country concerned.

8 Possible off-target effects

Off-target effects of breeding techniques are intensively studied as they are very important for the risk assessment. In the framework of the 2010 JRC study on NPBTs, an expert group evaluated intended changes in the genome and off-target effects of NPBTs. The latter are summarised below. However, it should be stressed that such off-target effects occur also in conventional breeding.

TECHNIQUE	Examples of possible off-target effects
ZFN-1	Non-specific binding can lead to non-specific DBSs, resulting in unintended mutations. This can lead to cytotoxicity.
ZFN-2	
ZFN-3	
ODM	The mutation rates achieved are usually low and are comparable to the rate of spontaneous mutations. Therefore spontaneous mutations may obscure the mutations of interest.
Cisgenesis and itnragenesis	Gene insertion may lead to interruption of open reading frames (ORFs) or creation of new ones or deletion of host DNA. Transfer of endogenous genes and regulatory elements to another plant can result in modified levels of expression and even gene silencing. Gene expression may be changed more extensively for intragenesis as new combinations of gene fragments are used.
RdDM	It is not clear for how many generations the effect of gene silencing by RdDM remains in the absence of the inducing construct. An unintended effect could therefore be the loss of silencing of the specific gene in the commercial product. Another potential unintended effect could be the silencing of genes with homologous promoter sequences. Alternatively, the production of other small RNAs from an hpRNA can occur that may regulate the expression of other genes not intended to be manipulated.
Grafting on GM rootstock	Macromolecules such as recombinant proteins, hormones and non-coding RNA (e.g. siRNA) can be transported from the GM rootstock of a graft to the scion where they can induce unintended effects, e.g. RNAi can lead to RNA-directed methylation of promoter regions, resulting in modified expression of the target genes. So mitotically and meiotically heritable (epigenetic) changes in gene expression that do not involve a change in the DNA sequence can occur.
Reverse breeding	Unintended effects could include the silencing of other homologous sequences in the genome as a result of the presence of the RNAi construct. This would not induce genomic changes, but could affect expression levels. Another unintended effect of the technique could be an incomplete suppression of meiosis. This would lead to some degree of meiosis and recombination, which are natural processes in plants.

TECHNIQUE	Examples of possible off-target effects
Agro-infiltration "sensu stricto **Agro-infection** **Floral dip**	While the aim is the transient and temporary expression of a coding sequence, the integration of T-DNA fragments into the genome of cells in the infiltrated area cannot be ruled out. This is true for agro-infiltration and for agro-inoculation/agro-infection. In the case of agro-infection, the spreading of the gene construct introduced into the viral genome is caused by systemic spreading of RNA viruses throughout the plant via plasmodesmata. Since the gene construct is spread via RNA molecules, they do not integrate into the plant genome.

9 Possibility to detect and identify crops developed by the technique

Availability of validated detection methods is a regulatory requirement for GMOs under the EU legislation. The possibilities for detecting and identifying crops produced with NPBTs were investigated as part of the study on "New plant breeding techniques: state-of-the-art and prospects for commercial development". For this evaluation an *ad-hoc* task force of experts was established.

Information concerning the genotype of plants can be obtained at different levels, e.g. at the level of DNA, proteins or metabolites. Modern analytical methods exist for all of these levels and the task-force discussed their applicability for the detection and identification of crops developed through NPBTs. The task-force concluded that DNA is the best target molecule for detecting and identifying unambiguously a change in the genetic material of plant and that amplification-based methods (polymerase chain reaction, PCR) are the most appropriate for the purpose. However, a certain minimum of information about the changed DNA sequence is required in order to allow the identification of a genetic modification.

The experts concluded that for crops derived through most of the NPBTs it is not possible to unambiguously differentiate them from crops resulting from natural mutation or conventional breeding.

TECHNIQUE	yes	no
ZFN-1		Detection with DNA-based methods would be possible provided some prior information on the introduced modification is available. But identification will not be possible because products cannot be distinguished at molecular level from products developed through other mutation techniques or occurring through natural mutations.
ZFN-2		
ZFN-3	Detection and identification are possible through the amplified based methods (PCR) currently used for detection, with the prerequisite that prior adequate DNA sequence information on the introduced modification is available. If there is no prior knowledge, the strategies used for detection of unknown GMOs may be applied to detect the large modifications resulting from ZFN-3. Identification will however not be possible without prior knowledge.	
ODM		See ZFN-1 and ZFN-2.
Cisgenesis and Intragenesis	Cisgenic/intragenic plants can be detected and identified as such when the event is known beforehand, i.e. when adequate information about the cisgenic/intragenic modification is made available. Event-specific primers can be developed to create a detection and identification method.	
RdDM		Specific gene silencing is obtained through DNA methylation and/or histone methylation in the chromatin but the DNA sequence itself is not modified. Since it is very difficult to differentiate between methylation occurring naturally and methylation through the deliberate use of a technique like RdDM, it can be concluded that identification of RdDM products is not possible, even with prior knowledge.
Grafting on GM rootstock		As the sequence of the non-GM scion is not modified, detection and identification of the GM rootstock on the basis of the harvested product is not possible.
Reverse Breeding		The end-products of reverse breeding are free of genetic modification-related DNA sequences because the homozygous parental lines are produced from double-haploid plants which are

TECHNIQUE	yes	no
		screened for the absence of RNAi construct during the breeding process. It is therefore not possible to distinguish products resulting from the use of reverse breeding technique from products resulting from conventional breeding.
Agro-infiltration "sensu stricto"		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. Detection and identification of products from agro-infiltration or agro-infection is therefore not possible.
Agro-infection		
Floral dip	In the case of floral dip, the aim is to select for stable integration into the germline, leading to a genetically modified plant, which means that detection and identification are possible with the methods currently available for GMO detection (PCR) and also implies that adequate information needs to be available.	