



# Comparative regulatory approaches for groups of new plant breeding techniques

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**This manuscript provides insights into ongoing debates on the regulatory issues surrounding groups of biotechnology-driven ‘New Plant Breeding Techniques’ (NPBTs). It presents the outcomes of preliminary discussions and in some cases the initial decisions taken by regulators in the following countries: Argentina, Australia, Canada, EU, Japan, South Africa and USA. In the light of these discussions we suggest in this manuscript a structured approach to make the evaluation more consistent and efficient. The issue appears to be complex as these groups of new technologies vary widely in both the technologies deployed and their impact on heritable changes in the plant genome. An added complication is that the legislation, definitions and regulatory approaches for biotechnology-derived crops differ significantly between these countries. There are therefore concerns that this situation will lead to non-harmonised regulatory approaches and asynchronous development and marketing of such crops resulting in trade disruptions.**

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## Introduction

Legislation regulating organisms, including plants, developed through biotechnology was introduced in the European Union and other countries in the late 1980s or early 1990s in preparation for the cultivation and commercialisation of transgenic plants. Definitions of what constitutes a Genetically Modified Organism (GMO) were also developed at this time and are still applied without up-dates in many of the countries. Therefore, biotechnology-driven 'New Plant Breeding Techniques' (NPBTs) which have been developed more recently may cause problems for regulators as it may not be clear whether plants developed using these techniques fall under the current GMO legislation.

A study carried out in 2010 [18,19] showed that plants produced by some of these NPBTs are at an advanced stage of development. In most of the countries conducting field trials of GM plants requires authorisations or notifications. Therefore breeders whose genotypes produced by NPBTs have reached this stage of development are contacting regulators for advice on the regulatory status of their products vis-à-vis the GMO legislation. In cases where a GM approach is not acceptable for a company due to the high costs associated with risk assessment and authorisation or because GMOs are not accepted by their customer base, they may in fact seek such clarification at an early stage of product development.

A workshop organised by the Institute for Prospective Technological Studies (IPTS) in September 2011 with participants from Argentina, Australia, Canada, European Union, Japan and South Africa showed that regulators in different countries currently assess NPBTs technique-by-technique or even event-by-event [20]. (The list of participants and the agenda of this workshop are included in [supplementary data 1](#)). Taking into account discussions at this workshop we suggest in this manuscript a structured approach to make the evaluation more consistent and efficient.

A similar approach was used by Schaart et al. [29] in a study comparing the consequences of new biotechnology-based plant breeding techniques in comparison to conventional plant breeding.

As a first step in developing such an approach we established a list of nine criteria, which we regard as important for categorising and evaluating NPBTs (see [Box 1](#)). Criterion 1 deals with the rationale for plant breeding including the effect achieved. Criteria concerning the method of application (2 and 3), the process (4 and 5) and the product (6–9) were also established. We have evaluated seven NPBTs based on information from a previous JRC study [11,18,26] according to these criteria ([supplementary data 2](#)). The criteria used appear to be relevant for all countries with specific legislation for GM crops, e.g. for Argentina, Australia, European Union, Japan and South Africa.

Supporting data 2 shows that structuring according to these criteria will lead to ambiguous results for a few of the techniques and indeed for some of the criteria the outcomes may remain ambiguous for most of them. This situation is further complicated by the fact that several of the techniques will potentially be used in combination. Flow charts developed for categorising NPBTs on the basis of a straightforward definition of what constitutes a GMO or even on components of a more complex definition may be possible. However, the discussion of regulatory approaches taken by several countries with different legislation and definitions for biotechnology-derived plants is very complex. For the discussions in this paper we have grouped techniques according to similarities in their approach and use.

[Box 2](#) shows the five groups chosen. Group 1 consists of techniques which achieve site-specific mutations. Group 2 comprises techniques which deploy only genes from the plant species itself or cross-compatible species. Techniques which do not lead to changes in the genome include the growing group of breeding techniques, which use a transgenic inducer line (group 3). In such cases a transgenesis step is used during the breeding process but the transgene is segregated out during further breeding and is therefore not present in the final product, that is, it becomes a negative segregant. Group 4 includes grafting techniques and group 5 techniques use agro-infiltration.

### BOX 1

#### List of criteria relevant for categorising and evaluating NPBTs

##### Criteria and rationale for application of the technique

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

##### Method of application

2. Molecules deployed
3. Plant tissues or cells manipulated and method of delivery

##### Process

4. Process at molecular level following delivery of technology into the cell
5. Intermediate (transgenic) plant produced – transgene segregated out during final breeding steps or not

##### Product

6. Nature of heritable change in the genome if any
7. Changes developed through the NPBT achievable through conventional breeding or occurring naturally?
8. Possible off-target effects
9. Possibility to detect and identify crops developed by the technique

**BOX 2****Groups of NPBTs**

Group 1:	<b>Site specific mutagenesis</b>	Zinc Finger Nuclease (ZFN) technique Meganuclease (MN) technique Transcription Activator-Like Effector Nuclease (TALEN) technique Oligonucleotide-Directed Mutagenesis (ODM)
Group 2:	<b>Cisgenesis and Intragenesis</b>	Cisgenesis Intragenesis
Group 3:	<b>Breeding with transgenic inducer line</b>	RNA-dependent DNA methylation (RdDM*) Reverse breeding Accelerated breeding following early flowering
Group 4:	<b>Grafting techniques</b>	Grafting on GM rootstock
Group 5:	<b>Agro-infiltration techniques</b>	Agro-infiltration 'sensu stricto' Agro-infection Floral dip

\* RdDM leads to changes in the methylation status of the genome (epigenetic effect) but not to changes in the DNA sequence. Our view of a genome change is confined to changes in the DNA sequence.

**Comparison of GMO and related definitions**

From the countries participating in the workshop, the EU, South Africa, Australia, Argentina and Japan have introduced specific GMO legislation, which also includes a GMO definition. The EU definition is included in Directive 2001/18/EC on the deliberate release into the environment of genetically modified organisms [6]. A GMO is defined as an 'organism, with the exception of human beings, in which the genetic material has been altered in a way that does not occur naturally by mating and/or natural recombination'. The Annexes of the Directive include three lists of techniques, (i) techniques of genetic modification, including recombinant nucleic acid techniques, (ii) techniques not considered to result in a genetic modification such as in vitro fertilisation, natural processes like conjugation, transduction, transformation and polyploidy induction and (iii) techniques of genetic modification yielding organisms to be excluded from the Directive including, for example, mutagenesis. (The full wording of the GMO definitions is provided in [supplementary data 3.](#))

In South Africa, the Genetically Modified Organisms Act provides the GMO definition and a list of techniques where the Act shall apply and a further list where the Act shall not apply. The South African participant in the workshop confirmed that the definition is very similar to the EU definition (although there are some differences in the wording) and that similar problems arise when applying the definition to NPBTs.

In Australia, the Gene Technology Act 2000 provides the GMO definition and the Gene Technology Regulations 2001 include a list of techniques that are not considered to be gene technology and a list of organisms that are not genetically modified organisms. The wording of the definition and the contents of the lists show significant differences when compared with the EU definition. From the list of organisms that are not genetically modified organisms the Australian participant regarded one point as specifically important for the discussion of NPBTs: "A mutant organism in which the mutational event did not involve the introduction of any foreign nucleic acid (that is, non-homologous DNA usually from another species)".

In Japan, the GM definition in the Cartagena Domestic Law follows the definition of the Cartagena Protocol of 'living modified organisms resulting from modern biotechnology'. This means that the Law covers living organisms produced by (a) modern biotechnology such as recombinant DNA technology including self-cloning and/or recombinant DNA techniques using genetic material (host, vector and foreign genes) derived from an organism between which natural gene exchange is possible ('natural occurrence') and (b) techniques for fusing of cells of organisms belonging to different taxonomic families ('fusion techniques beyond taxonomic family'). Possible exemptions for organisms obtained by self-cloning and/or 'natural occurrence' are assessed and decided on a case-by-case basis. In this context exemption means that the organism produced falls under the Cartagena Domestic Law; however the requirements of the law are not applied to the specific organism.

Argentina uses two complementary criteria when defining GMOs, (i) the definition of products of 'modern biotechnology' as used in the Cartagena Protocol and (ii) the definition of 'event' in the national legislation. In the case of ambiguity, the definition of 'event' is decisive.

In Canada the Regulatory Framework for Biotechnology established the principles that apply to products of biotechnology. It was decided to continue using the existing legislation and that products derived through biotechnology are to be treated as any other novel product. This means that regulation is triggered by the novel trait of the product (plants with novel traits, PNTs), novel feeds and novel foods and not by the process via which the trait is introduced. The assessment of PNTs is based on science and decided on case-by-case basis. When PNTs are used as food or feed, the legislation for novel food and feed will also apply.

Regulators from the United States (USA) were not represented in the workshop. However, some of their decisions concerning NPBTs are available on the USDA (United States Department of Agriculture) website [33,34]. Like Canada, the USA decided that no specific legislation was required for regulating plants derived from biotechnology. The USDA regulates the environmental release of certain genetically engineered organisms, which are, or are

believed to be, plant pests under the Plant Protection Act. GM plants are regarded as a plant pest when genes from plant pests are introduced. As transgenic approaches frequently use *Agrobacterium* as a vector and/or genes from soil bacteria (e.g. antibiotic resistance genes) or viral promoter sequences (e.g. 35S promoter from cauliflower mosaic virus), most transgenic plants fall under this definition and consequently under the oversight of USDA.

The USDA decisions presented in the text below are restricted to evaluations under the Plant Protection Act, which regulates importation, interstate movement, or environmental release of crops regarded as plant pests. If the USDA concludes that a plant does not fall under the oversight of the Act then field trials can be carried out without the need for a permit or notification process. However, depending on the nature and the intended use the plant may still be subject to other regulatory authorities such as EPA (Environmental Protection Agency) and FDA (Food and Drug Administration). Furthermore these decisions apply only to the specific plants or specific applications of the techniques (as described in the application by the breeder or in the USDA letter respectively).

### Groups of NPBTs

In the following sections we discuss the NPBTs according to the groups suggested in [Box 2](#). We provide information and considerations for the regulation from [supplementary data 2](#) where relevant for the regulatory discussion and from the discussions in the workshop. We also present results from two expert groups which were established in the framework of the JRC study carried out in 2010 [18,19] and which evaluated the changes in the plant genome after application of NPBTs [11]. Issues related to detection and identification of plants obtained through NPBTs were also evaluated [26]. Finally we summarise the regulatory approaches (or preliminary discussions) for NPBTs in the countries represented in the workshop together with advice given by USDA to stakeholders and posted on their website [33,34].

#### Group 1: site-specific mutagenesis

Mutagenesis using chemicals or irradiation was introduced in plant breeding in the late 1920s. Mutagenised varieties are widely used in conventional agriculture across the world and the FAO/IAEA Mutant Variety Database lists more than 3000 varieties bred through mutagenesis [12]. Whereas these approaches result in random mutations in the genome, in more recent years new techniques have been developed which deliver site-specific mutations. Gene-targeting started in the late 1970s with applications on microorganisms [22] and since then gene-targeting techniques have been widely used with all kind of cells, including human cells.

The initial applications on plants are more recent and the first papers on the use of Oligonucleotide-Directed Mutagenesis (ODM) were published in the late 1990s. This approach of targeted mutagenesis was followed by other techniques including Meganuclease (MN; [14]), Zinc Finger Nuclease (ZFN; [27]) and the use of Transcription Activator-Like Effector Nuclease (TALEN; [25]). The ODM, ZFN and MN techniques have already been adopted by plant breeders and the first crops produced by ODM and ZFN techniques are at an advanced stage of development [18]. The development of TALEN technology started only recently.

### Technology

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 a few base pairs. The oligonucleotides target the homologous sequence in the genome of the host and induce site-specific nucleotide substitutions, insertions or deletions via the natural repair mechanisms of the cell.

MNs are natural proteins that cleave DNA in a site-specific way [7]. Zinc finger proteins (ZFs), and Transcription Activator-Like Effectors (TALEs), which occur in microorganisms are transcription factors which modulate gene expression. ZFs and TALEs can be converted into site specific 'DNA scissors' by fusing them to an endonuclease (usually FokI). Although these molecules occur naturally, molecules which are designed and engineered for high binding specificity are usually used for gene-targeting [4].

In reality ZFN, TALEN and MN are all site-directed nuclease (SDN) technologies and can each be used to introduce the same changes in the genome. SDNs function usually in pairs each recognising through the DNA binding domain an opposite DNA strands although there are cases where they function as monomers (e.g. ISceI). The DNA cleaving domain produces a site-specific double strand break (DSB) which stimulates the cell's repair mechanisms. DSBs are repaired via non-homologous end joining (NHEJ), or in the presence of a repair template via the homologous recombination (HR) pathway.

In the absence of a repair template, a totally random repair by NHEJ at the site of the double strand break leads to non-specific mutations such as nucleotide changes, deletions or insertions of one or more base pairs (SDN-1 technique). However, in addition to the nuclease a short repair template can be introduced which is homologous to the insertion site except for one or more specific nucleotide sequence changes to be introduced via homologous recombination (SDN-2 technology). The mutations introduced are those present in the repair template. Thus both the target site of the DSB and the introduced mutation are defined. These technologies can also be used to target more extensive changes to DNA sequences, including the insertion/replacement of entire genes and potentially also for gene stacking (e.g. for resistance genes). This is achieved by delivering a repair template consisting of the gene of interest flanked by DNA homologous with the target to the plant cell along with the SDN vector (defined as SDN-3 technology). The resulting plants are different from traditional transgenic plants only insofar as the insertions are site-specific. SDN-3 techniques can also be carried out with cisgenes, although due to the restricted gene pool, suitable genes may not always be available (see group 2).

#### Considerations for regulation

At the time of the introduction of GMO legislation, mutagenesis (which then could only be achieved through irradiation or chemicals) was regarded as a 'conventional breeding technique' because of the long history of safe use. Therefore crops produced by 'conventional' mutagenesis do not fall under the GM legislation in different countries.

Some of the participants in the workshop suggested that development from 'conventional' random mutagenesis through

irradiation or chemicals to modern targeted mutagenesis through ODM, ZFN, MN or TALEN techniques could be seen as continuum, which would exclude these techniques from GM legislation.

It was stressed that products of the modern mutagenesis techniques could also in principle be achieved by random mutagenesis or by spontaneous natural mutations. The considerably higher costs for selecting plants produced by random mutagenesis might outweigh the higher regulatory costs for targeted mutagenesis should the latter be regulated as a technique of genetic modification.

Crops produced by targeted mutagenesis are not distinguishable from crops derived through random mutagenesis. An expert group evaluating the possibilities of detecting and identifying crops derived through NPBTs concluded that for organisms modified by ODM or ZFN-1 and ZFN-2 techniques detection with DNA-based methods would be possible provided some prior information on the introduced modification is available [26]. However, these organisms could not be distinguished from other chemically or naturally mutated organisms at the molecular level, which means that identification is not possible. This conclusion will also apply to products developed with MN and TALEN.

Some of the issues have to be considered which clearly distinguish the new approaches from 'conventional' mutagenesis. To date, SDN techniques have primarily used plasmid vectors to introduce the gene encoding the SDN, although SDN can also be introduced into the plant cell as mRNA and the possibility of delivering SDN directly as proteins has been demonstrated in mammalian cells [10]. SDN genes delivered into the cell are integrated into the plant as a transgene or transiently expressed in the cells. In the case of SDN-2 technique a further DNA sequence is delivered as part of the DNA construct, which acts as the repair template. Transiently expressed DNA sequences degrade and plants carrying the inserted SDN gene are segregated out during the further breeding process. However, the use and possible integration of foreign DNA sequences may be relevant for regulatory decisions depending on the GMO definition and its interpretation.

#### *Regulatory discussions and decisions*

In the workshop organised at JRC-IPTS, regulatory approaches for crops derived through ODM and SDN techniques (ZFN and MN techniques specifically) were discussed by the participating experts. As the development of TALENs for use in plants is at an early stage the regulatory aspects of this technique have not yet been discussed specifically by regulators. Because ZFNs, TALENs and MNs are all SDNs and can all be used to introduce the same changes in the genome ZFN and MN discussions are likely to apply also to TALENs.

Experts from most of the participating countries regard it as very probable that the SDN-1 techniques whereby no template sequences are introduced will be classified as non-GM. The EU has not yet concluded its assessment. Products of the SDN-3 techniques are products of recombinant DNA techniques (GMOs).

For SDN-2 techniques or ODM (where both the target site of the DSB and the introduced mutation are defined), it generally appears to be unclear which kind, and specifically what size, of change obtained by the technique should result in the classification of the product as a GMO or a non-GMO. The representatives of Argentina specified that in their country SDN-2 techniques in which coding

sequences are introduced or open reading frames (ORFs) are modified will most probably be treated on a case-by-case basis. The representative of the Australian Office of the Gene Technology Regulator informed the workshop that the Office has advised that products of the ZFN-2 technique and ODM are likely to be considered as GMOs if any nucleotide is changed.

Other experts, however, stressed that products obtained by SDN-1 and SDN-2 techniques cannot be distinguished from crops derived through mutagenesis induced by chemicals or irradiation and, therefore, should not be regulated as GMOs.

Complementary to the workshop, decisions made by the USDA concerning crops derived by targeted mutagenesis are publically available. Letters from USDA to companies who contacted them concerning the regulatory status of crops produced by site-specific mutagenesis are published on the USDA web page [33,34]. A letter from 2004 states that under the current regulations, USDA has no authority to regulate products created by mutagenesis techniques such as genoplasty (genoplasty is a synonym of ODM.) Concerning plants derived by MN techniques (letter from 2011), USDA concluded that plants containing targeted gene deletions will not, in most cases, be regulated articles under the Plant Protection Act, unless the engineered plant is already a plant pest or if MN is delivered into the plant using a plant pest. For applications where template DNA molecules are used (this corresponds to SDN-2 and SDN-3 as defined in this paper), the Agency will consider case-by-case enquiries regarding the regulatory status of the plants. Similar conclusions were drawn for plants produced by ZFN technology with or without template DNA molecules (letters from 2010 and 2012 [33]).

#### *Group 2: cisgenesis and intragenesis*

More than 10 years ago, the concept of cisgenesis/intragenesis was introduced in plant breeding. Cisgenesis and intragenesis exclusively apply DNA sequences from the plant species itself or cross-compatible species by contrast to transgenesis which deploys DNA sequences from any organism. Several different terms are used in literature such as autotransgenesis [1], intragenesis [28] or cisgenesis [30]. In addition, experts define these terms in different ways.

For this manuscript we define cisgenesis as genetic modification of a recipient organism with a gene from a crossable organism (same species or closely related species). This gene includes its introns, is flanked by its native promoter and terminator without any rearrangements and is inserted in the normal sense orientation. In the case of intragenesis, the inserted DNA can be a new combination of DNA sequences from the species itself or from cross-compatible species, which can be inserted in sense or anti-sense orientation.

#### *Technology*

Cisgenesis and intragenesis deploy the same gene transfer technologies as transgenesis. *Agrobacterium*-mediated transformation is the most widely used method for delivery of the gene construct, although biolistics is also used.

#### *Considerations for regulation*

Whereas the concepts of cisgenesis and intragenesis are only used in the context of plant breeding and did not exist at the time of the introduction of first GM legislation, an analogous process used



with microorganisms, the so-called 'self-cloning' was already well-established. Self-cloning means the re-insertion of nucleic acid sequences removed from an organism into cells of the same species or closely related species which can exchange genetic material by natural physiological processes. Self-cloning of micro-organisms is exempted from the GM legislation, for example, in Japan, Australia (although here the term 'self-cloning' is not used) and the EU (under Directive 2009/41/EC on the contained use of genetically modified micro-organisms). Therefore experts discussed the appropriateness of exempting cisgenesis/intragenesis on the same basis.

Cisgenesis and intragenesis use the same technology as transgenesis (with the exception of sources of the gene to be transferred) and therefore fall under the definition of a technique of genetic modification unless, as already indicated, the use of cisgenes is exempted (see the discussion of the Australian GMO definition above). Some of the definitions of GMOs specify as a criterion that the achieved modification does not occur naturally through mating or natural combination (e.g. EU and South African definitions). Therefore, experts discussed if plants resulting from cisgenesis and intragenesis applications could also be achieved by conventional breeding techniques such as sexual crosses within species, or in the case of more distant crosses, bridge crosses or wide crosses using embryo rescue. Cisgenesis (as defined above) deploys genes with their own regulatory elements and introns and in their natural configuration. In principle, cisgenic 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 [13], it has to be decided on a case-by-case basis, whether plants derived by this approach can also be produced by conventional breeding. Specific vectors have been constructed for cisgenic/intragenic approaches, which use DNA sequences originating from the same species or related species to insert the target genes. This so-called P-DNA approach avoids the insertion of bacterial DNA into the plant genome [2].

Intragenesis (as defined above) also deploys combinations of fragments from different genes from the plant species itself or from a cross-compatible plant, for example, combining coding sequences from one gene with regulatory elements from other genes. Genes can be inserted in their natural sense and also antisense configuration. It is extremely unlikely that these changes can be mimicked using a conventional breeding approach. More detail on the comparative changes in the genome introduced by cisgenics and intragenics compared with both transgenesis and conventional breeding can be found in an EFSA opinion [5].

Cisgenic and intragenic plants can be detected and identified when adequate information about the modification is available [26]. Polymerase chain reaction technique (PCR) will be the most suitable method for analysis. Event-specific primers can be developed to create a detection and identification method. Without prior knowledge provided by the producer, cisgenic and intragenic crops cannot be identified as such. Screening for cisgenic/intragenic plants is not possible due the absence of certain common elements (e.g. the 35S promoter or NOS terminator are commonly used to screen for unknown GMOs).

### *Regulatory discussions and decisions*

All participants in the workshop agreed that in their countries intragenesis will most probably be treated in the same way as transgenesis. Cisgenesis is also expected to be classified as a technique of genetic modification with the exception of specific approaches of cisgenesis in a few countries. The Australian participant in the workshop stated that cisgenesis with a very narrow definition (introduced gene from the same species and without any rearrangements, no foreign DNA, and no T-DNA border sequences) would probably not fall under the Australian GMO definition. However, the Office of the Gene Technology Regulator has not yet dealt with such a case. The experts noted that applications of cisgenesis falling under this narrow definition (obtained through a biolistic approach) are rare. Also the expert from South Africa indicated that, according to preliminary discussions in her country, some of the approaches of cisgenesis might be treated as non-GM. The Japanese expert confirmed that crops obtained by cisgenesis are currently treated as GMOs in his country. Also the Argentinean experts group concluded that cisgenesis should not be treated any differently from transgenesis.

USDA was approached by a plant breeder concerning the regulatory status of a grapevine transformed by an 'ingenic or cisgenic' approach (which corresponds to the definition of intragenesis used in this paper). The plant which carries a grapevine-derived anthocyanin regulatory gene and grapevine-derived regulatory elements is not considered to be a regulated article under the Plant Pest Act (letter from 2012 [33]).

### *Group 3: breeding with transgenic inducer line*

This is a heterogeneous group of techniques, which use transgenesis as an initial step in the breeding process. However, the transgene is segregated out during further breeding and is therefore not present in the final product (negative segregant). This paper describes three of these techniques, which are already at an advanced stage of development. Results of a survey carried out in 2010 showed that additional techniques, which fall within this group are under development [18].

Development of RNA-dependent DNA methylation (RdDM), which can be used to achieve gene silencing, started more than ten years ago [24]. The technique is mainly used on model plants and genes and the applicability for the breeding of commercial crops is questionable because of the instability of the traits achieved (see discussion below).

The technique of reverse breeding allows homozygous parental lines to be produced for the production of F1 hybrids faster than with conventional breeding. It was developed by a Dutch company which is holding two patents [3,36]. Crops developed by this technique are still in the research phase.

The development of accelerated breeding following early flowering started around ten years ago [38]. Until recently research efforts had not led to the production of fertile, viable or normal gametes and progeny [9]. However, the technique has been used successfully in plum breeding [35].

### *Technology*

For all the techniques within this group a transgene encoding an RNAi construct or a dominant-negative protein is inserted 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 leads to an effect such as suppression of the meiotic recombination or early flowering. The inducer transgene is segregated out during further breeding and is therefore not present in the final product (negative segregant).

RdDM is achieved by insertion of genes (transgenesis) encoding RNAs which are homologous to the promoter regions of the target gene. Transcription of the gene leads to double stranded RNAs, which after cutting by specific enzymes into sRNAs (small RNAs) induce 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 methylated status can be inherited over several generations, the epigenetic effect will eventually fade out.

Reverse breeding comprises several consecutive steps. First a transgene encoding RNA interference (RNAi) sequence is delivered to explant material 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 into homozygous diploid plants (doubled haploid technology) under suitable cell culture conditions. In a final step plant pairs that do not contain the transgene and whose hybridization reconstitutes the elite heterozygous line are selected.

Approaches other than 'breeding with transgenic inducer line' potentially can be used for reverse breeding, for example, it is possible to use a natural process called second division restitution in meiosis to obtain homozygous parental lines. This technique is called 'near reverse breeding' as the reconstitution is 'near complete' and the reconstituted hybrids will genetically differ to some extent from each other as well as from the original starting hybrid [37]. The discussions in the IPTS workshop focused on reverse breeding using a transgenic inducer line only. Therefore the conclusions from the workshop do not apply to alternative approaches for which the actually used approach will be relevant for the categorisation vis-à-vis the GMO legislation.

Early flowering can be achieved by reducing juvenility/vegetative maintenance factors by gene silencing. This can reduce the time of breeding cycles considerably, especially for perennial crops [15]. Alternatively, the over-expression of a flower initiation related transcription factor gene can induce early flowering [8]. In an initial step, transgenic plants are produced using gene silencing or over-expression constructs. The effect of early flowering will be present for several breeding cycles until the breeding target is reached. In a final breeding step the transgene used for induction of early flowering will be crossed out.

#### Considerations for regulation

According to available publications, current applications of the three techniques described above deploy a plasmid vector carrying a transgene. Genes delivered into the cell are integrated into cells of explants which are regenerated into transgenic plants. However, during the final breeding process, plants carrying the inserted transgene are segregated out. Consequently the commercialized plant is not itself transgenic but is a progeny of a GM plant. From

most of the GMO definitions it is not clear if progeny of a GMO which no longer contains the transgene falls under the GMO legislation. The Australian definition is more specific by defining that progeny of a GMO, which has inherited the introduced trait is a GMO.

Transcriptional gene silencing leads to changes in the methylation status of the DNA. Although this process potentially constitutes a new trait this is not linked to a change in the sequence of base pairs, but constitutes an epigenetic effect. Although this effect can be inherited over several generations it will eventually fade out. This effect probably will not be classified as genetic modification because the DNA sequence is not changed and/or the lack of stability which are criteria for the classification as GMO according to most of the definitions.

Also the results of the experts group who evaluated the possibility of detecting and identifying these changes should be mentioned [26]. Because it is very difficult to differentiate between 'natural' methylation and that induced through the deliberate use of a technique like RdDM, the identification of RdDM products is not possible, even with prior knowledge. 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 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. The possibility of detecting and identifying crops produced through accelerated breeding following early flowering was not evaluated. However, by analogy the conclusion that identification is not possible will also apply to this technique.

#### Regulatory discussions and decisions

The regulatory status of negative segregants appears to be unclear in most of the countries represented in the workshop. The experts from Argentina stated that, according to a preliminary discussion in their expert group, negative segregants should be excluded from the GMO legislation. The participant from Australia stated that a negative segregant is not regarded as falling under the GMO definition of his country if no introduced trait is inherited. However, if an introduced trait is inherited (e.g. gene silencing generated by RdDM) then the progeny may fall under the Australian definition of a GMO even when the introduced DNA is not inherited. However, a submitted application concerning this issue has not yet been dealt with. The EU and South Africa still have to conclude on the classification of negative segregants. The participant from Japan stressed the importance of proving the absence of inserted DNA sequences.

The special case of RdDM, where the methylation of certain regions of the DNA remains after segregating out the inserted gene, was also discussed. Here a more general problem is prevalent. The effect of gene silencing fades out in the following generations. The Canadian and Argentinean representatives indicated that because of this instability of expression it is unclear how crops with such traits would be treated under the current regulatory framework. This question needs to be addressed.

USDA was contacted by plant breeders concerning the regulatory status of 'null-segregant' (negative segregant) lines derived from genetically modified early flowering parents (plums) and

parents (sorghum) transformed by an RNAi transgene to down-regulate the expression of a native plant gene. In letters from 2011 and 2012 [33], USDA replied that they do not consider the described 'null segregant' lines to be regulated articles.

#### Group 4: grafting techniques

Grafting on GM rootstock combines grafting which has been used since ancient times and transgenesis. Research on grafting on GM rootstock started more than 20 years ago [16,21].

Grafting is a practice widely used for fruit trees and also for some vegetables such as potatoes and eggplants. Grafting combines the desired properties of a rootstock with those of the donor scion. There are many potential benefits from the use of GM rootstock in grafting including enhanced root performance (disease resistance, better rooting, nutrient and water acquisition).

#### Technology

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. In principle there are three possible approaches for producing grafts with GM components (non-GM scion on GM rootstock, GM scion on non-GM rootstock and GM-scion on GM-rootstock). The case of interest for the regulatory discussion is grafting of a non-GM scion on a GM rootstock and therefore we restrict the following discussions to this case. Plants used for grafts can be transformed by transgenesis, cisgenesis or other technologies. The vascular cambium tissues of the stock and scion plants must be placed in contact with each other and vascular connection has to take place between the grafted tissues.

#### Considerations for regulation

In the case of grafting on GM rootstock, the chimeric plant contains transgenic tissue and therefore will be regarded as GM plant. As the scion is not transgenic, the question arises if crops (fruits) and seeds and the progeny thereof should be regarded as GM.

One potential issue is the possibility that transmission of recombinant DNA from the root stock to the scion could induce changes to the genome in the non-transformed tissues following grafting. However, there is little evidence that this is an issue. Genetic exchange appears to be restricted to graft sites only. Stegmann et al. [31] showed that flowers and fruits from a non-GM scion did not contain recombinant DNA sequences from the rootstock.

Another potential issue relates to changes to the scion resulting from the transmission of macromolecules and/or metabolites generated as a result of the genetic modification. It is known that 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 an effect. It is also known that RNAi can lead to RNA-directed methylation of promoter regions, resulting in modified expression of the target genes (see section RdDM above). So, although the offspring generated from the product of a graft can be regarded as non-GM, mitotically and meiotically heritable (epigenetic) changes in gene expression that do not involve a change in the DNA sequence can still occur [23].

Experts evaluating the possibility of detection and identification of grafts with GM rootstock concluded that detection and identification of the GM rootstock on the basis of the harvested

product is not possible as the DNA sequence in the genome of the non-GM scion is not modified [26]. If the harvested product was originating from a scion that was grafted on a GM-rootstock, it can be expected that the scion's transcriptome might differ from a graft on a non-GM rootstock. In principle this could be analysed by transcriptome analysis. However, this cannot be performed routinely. In the whole chimeric plant, including the GM rootstock, detection and identification will be possible.

#### Regulatory discussions and decisions

As for grafting on GM rootstock, the experts participating in the workshop stated that the rootstock is clearly GM and that an approval is required for the plant's release into the environment. Scientific questions still need to be answered, especially concerning the possible migration of molecules from the rootstock to the scion. In Japan fruits from such a graft are treated as GMOs (taking into account the possible trafficking of proteins and metabolites). However, the seeds and progeny are regarded as non-GM. The Argentinean group of experts concluded (preliminary opinion) that the fruits of these grafts should be assessed on a case-by-case basis. In Australia, fruits from grafts on GM rootstock will most probably not be regarded as GMOs, but may be classified under the food legislation (Australia New Zealand Food Standards Code) as 'food produced using gene technology' and may therefore require a pre-market safety assessment. In South Africa it was concluded that the use of the fruits should be taken into account for the assessment.

#### Group 5: agro-infiltration techniques

Agro-infiltration has been used for more than 10 years [17] and the technique is versatile in terms of possible applications. It can be used to study the functionality of a gene construct, to evaluate the impact of gene knock-outs, among others. In plant breeding it is used to search for plants with certain traits, for example, resistance genes. It can be also used in plant molecular farming for the production of recombinant proteins in infiltrated plants.

#### Technology

Depending on the tissues and the type of constructs infiltrated, three types of agro-infiltration can be distinguished. In the case of agro-infiltration 'sensu-stricto' 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. The technology is applied to intact plants or detached plant parts. 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 the 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 sp.* containing a T-DNA construct to stably transform the female gametocyte and obtain GM seeds. Plants obtained from these seeds do not differ from GM plants obtained by other transformation methods. Plants derived through these techniques are clearly GMOs and they were not discussed further during the workshop. Floral dip can use transgenes or cisgenes. However, the application of floral dip uses *Agrobacterium* as a carrier and therefore in the case of stable integration in the genome, insertion of T-DNA border



sequences has to be expected. Some experts exclude approaches which lead to T-DNA border sequences in the host genome from the definition of cisgenesis and intragenesis (see the discussion in group 2).

#### Considerations for regulation

In the case of agro-infiltration and agro-infection the aim is the transfer and temporary expression of a coding sequence (usually for experimental purposes). However, with agro-infiltration integration of T-DNA fragments into the genome of cells in the infiltrated area cannot be excluded. In the case of agro-infection, the gene construct is spread throughout the plant by RNA viruses moving through plasmodesmata. Because the gene constructs are spread via RNA molecules they do not integrate into the plant genome.

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 [26]. 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.

#### Regulatory discussions and decisions

Scientific questions still have to be addressed for agro-infiltration too, for example relating to the absence of *Agrobacterium* or if integration of the gene takes place. In Australia and Argentina progeny of infiltrated plants will most probably not be regarded as GMO if no *Agrobacterium* is present and no gene is integrated. In South Africa, agro-infiltration is used in research and therefore the regulatory status is under discussion. However, no final view has been reached. The Japanese participant stressed the interest of researchers and breeders in this technique in his country.

#### Conclusions

Biotechnology based 'new plant breeding techniques' which have been developed since the worldwide introduction of GM

legislation and definition provide challenges for regulators as their classification under GMO legislation is not clear in many cases. Comparison of the situation in seven countries (Argentina, Australia, Canada, EU, Japan, South Africa and USA) shows that legislation, definitions and regulatory approaches for biotechnology derived crops differ significantly between these countries. Decisions are usually taken on a technique-by-technique or even a case-by-case basis for any one technique and there has been variable progress in decision making on a country by country basis. Some of the countries are already concluding their positions whereas others are still in the process of evaluation or have only recently started preliminary discussions.

Preliminary results of the discussions and first decisions indicate that deviating opinions can be expected and that applications of the same technique, or a very similar technique, in breeding will result in different classification outcomes (GMO or non GMO) depending on the country concerned.

Differences in the legislation of biotechnology derived plants and in the regulatory process have resulted in asynchronicity in approvals of new crops. In some cases traces of GMOs have been detected in countries where they had not been approved which has led to trade disruptions [32]. A global discussion of the regulation of biotechnology derived crops appears to be indispensable to avoid similar cases in the future. To stimulate this discussion this paper and its [supplementary data 2](#) provide suggestions for a structured approach to assist the required dialogue. The international workshop on comparative regulatory approaches for new plant breeding techniques, the results of which are discussed in this paper, was highly appreciated by the attending regulators and researchers as a first international (although informal) platform for such discussions.

#### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.nbt.2013.02.004>.

#### References

- [1] Beardmore JA. Transgenics: autotransgenics and allotransgenics. *Transgenic Research* 1997;6:108.
- [2] Conner A, Barrell P, Baldwin S, Lokerse A, Cooper P, Erasmussen A, et al. Intragenic vectors for gene transfer without foreign DNA. *Euphytica* 2007; 154:341–53.
- [3] Dirks, RHG, Van Dun, CMP, Reinink K, De Wit, JPC. Reverse Breeding, WO/03/017753, Rijk Zwaan Zaadelt en Zaadha [NL]; 2001.
- [4] Durai S, Mami M, Kandavelou K, Wu J, Porteus MH, Chandrasegaran S. Zinc finger nucleases: custom-designed molecular scissors for genome engineering of plant and mammalian cells. *Nucleic Acids Research* 2005;33:5978–90.
- [5] EFSA. Scientific opinion addressing the safety assessment of plants developed through cisgenesis and intragenesis. *EFSA Journal* 2012;10(2):2561. <http://dx.doi.org/10.2903/j.efsa.2012.2561> [33 pp.].
- [6] EU. Directive 2001/18/EC of the European Parliament and of the Council of 12 March 2001 on the deliberate release into the environment of genetically modified organisms and repealing Council Directive 90/220/EEC – Commission Declaration – OJ L 106, 17.4.2001; 2001. p. 1–39.
- [7] Fajardo-Sanchez E, Stricher F, Pâques F, Isalan M, Serrano L. Computer design of obligate heterodimer meganucleases allows efficient cutting of custom DNA sequences. *Nucleic Acids Research* 2008;36:2163–73.
- [8] Flachowsky H, Hanke MV, Elo A, Sopanen T. BpMADS4 – a MADS Box gene of birch induces flowers on transgenic apple plants in vitro. *Acta Horticulturae* 2007;738:307–12.
- [9] Flachowsky H, Hanke MV, Peil A, Strauss SH, Fladung M. A review of transgenic approaches to accelerate breeding of woody plants. *Plant Breeding* 2009;128: 217–26.
- [10] Gaj T, Guo J, Kato Y, Sirk SJ, Barbas CF. Targeted gene knockout by direct delivery of zinc-finger nuclease proteins. *Nature Methods* 2012;9:805.
- [11] Glandorf B, de Loose M, Davies H. Evaluation of changes in the genome of plants through application of new plant breeding techniques. Annex 15. In: Lusser M, Parisi C, Plan D, Rodriguez-Cerezo E, editors. *New plant breeding techniques. State-of-the-Art and Prospects for Commercial Development*. JRC. JRC Technical Report EUR 24760; 2011. p. 141–55 [European Commission Joint Research Centre].
- [12] IAEA. 2000. <http://nucleus.iaea.org/sso/NUCLEUS.html?exturl=http://www-mvd.iaea.org/MVD/default.htm>.
- [13] Jacobsen E, Schaart J. Risk assessment of T-DNA borders from *Agrobacterium tumefaciens* in cisgenic crops. In: Letter to Minister Cramer (VROM) March 13. 2009. Department of Plant Breeding (WUR); 2009.
- [14] Kirik A, Salomon S, Puchta H. Species-specific double-strand break repair and genome evolution in plants. *EMBO Journal* 2000;19:5562–6.
- [15] Kotoda N, Wada M. MdTFL1, a TFL1-like gene of apple, retards the transition from the vegetative to reproductive phase in transgenic Arabidopsis. *Plant Science* 2005;168:95–104.
- [16] Lambert C, Bianco J, Garello G, Le Page-Degivry MT. Alteration of hormonal levels and hormone sensitivity by Ri T-DNA-transformation of apple cuttings. *Journal of Plant Physiology* 1998;153:677–83.
- [17] Lee RWH, Strommer J, Hodgins D, Shewen PE, Niu YQ, Lo RYC. Towards development of an edible vaccine against bovine pneumonic pasteurellosis using transgenic white clover expressing a *Mannheimia haemolytica* A1 leukotoxin 50 fusion protein. *Infection and Immunity* 2001;69: 5786–93.

- [18] Lusser M, Parisi C, Plan D, Rodríguez-Cerezo E. New plant breeding techniques: state-of-the-art and prospects for commercial development. JRC Technical Report EUR 24760; 2011 [European Commission Joint Research Centre].
- [19] Lusser M, Parisi C, Plan D, Rodríguez-Cerezo E. Deployment of new biotechnologies in plant breeding. *Nature Biotechnology* 2012;30:231–9.
- [20] Lusser M, Rodríguez-Cerezo E. Comparative regulatory approaches for new plant breeding techniques. Workshop proceedings. European Commission. JRC Technical Report EUR 25237 EN; 2012.
- [21] MacKenzie DJ, Tremaine JH, McPherson J. Genetically engineered resistance to potato virus-S in potato cultivar Russet Burbank. *Molecular Plant-Microbe Interactions* 1991;4:95–102.
- [22] Mahfouz MM, Li L. TALE nucleases and next generation GM crops. *GM Crops* 2011;2:99–103.
- [23] Martienssen RA, Colot V. DNA methylation and epigenetic inheritance in plants and filamentous fungi. *Science* 2001;293:1070–4.
- [24] Mette MF, van der Winden J, Matzke MA, Matzke AJM. Production of aberrant promoter transcripts contributes to methylation and silencing of unlinked homologous promoter in trans. *EMBO Journal* 1999;18:241–8.
- [25] Miller JC, Tan S, Qiao G, Barlow KA, Wang J, Xia DF, et al. A TALE nuclease architecture for efficient genome editing. *Nature Biotechnology* 2011;29:143–8.
- [26] NTTF. Report from new Techniques Task Force. New plant breeding techniques challenges for detection and identification. Annex 16. In: Lusser M, Parisi C, Plan D, Rodríguez-Cerezo E, editors. *New Plant Breeding Techniques. State-of-the-Art and Prospects for Commercial Development*. JRC. JRC Technical Report EUR 24760; 2011. p. 157–99 [European Commission Joint Research Centre].
- [27] Puchta H, Hohn B. Green light for green targeting in plants. *Proceedings of the National Academy of Sciences of the United States of America* 2005;102:19961–62.
- [28] Rommens CM. All-native DNA transformation: a new approach to plant genetic engineering. *Trends in Plant Science* 2004;9:457–64.
- [29] Schaart JG, Visser RGF. *Novel Plant Breeding Techniques – Consequences of new genetic modification-based plant breeding techniques in comparison to conventional plant breeding*. COGEM Research Report number 2009-02. The Netherlands Commission on Genetic Modification; 2009.
- [30] Schouten HJ, Krens FA, Jacobsen E. Cisgenic plants are similar to traditionally bred plants – international regulations for genetically modified organisms should be altered to exempt cisgenesis. *EMBO Reports* 2006;7:750–3.
- [31] Stegmann S, Bock R. Exchange of genetic material between cells in plant tissue grafts. *Science* 2009;324:222–8.
- [32] Stein AJ, Rodríguez Cerezo E. International trade and the global pipeline of new GM crops. *Nature Biotechnology* 2010;28:23–5.
- [33] USDA. [http://www.aphis.usda.gov/biotechnology/reg\\_loi.shtml](http://www.aphis.usda.gov/biotechnology/reg_loi.shtml).
- [34] USDA. [http://www.aphis.usda.gov/foia/efoia\\_list.php?path=/2011/Biotechnology%20and%20Regulatory%20Services%20\(BRS\)/11-089%20-%20Correspondence%20Concerning%20Regulatory%20Status%20of%207%20CFR%20Part%20340](http://www.aphis.usda.gov/foia/efoia_list.php?path=/2011/Biotechnology%20and%20Regulatory%20Services%20(BRS)/11-089%20-%20Correspondence%20Concerning%20Regulatory%20Status%20of%207%20CFR%20Part%20340).
- [35] USDAC <http://www.ars.usda.gov/is/AR/2011/mar11/breeding0311.htm>.
- [36] Van Dun CMP, Dirks RHG. *Near Reverse Breeding*, WO/2006/094773, Rijk Zwaan Zaadelt en Zaadha [NL]; 2005.
- [37] Van Dun CMP, Dirks RHG. *Near Reverse Breeding*, United States Patent Application Publication; Pub. No.: US 2008/0098496; 2008.
- [38] Wada M, Cao Q, Kotoda N, Soejima J, Masuda T. Apple has two orthologues of FLORICAULA/LEAFY involved in flowering. *Plant Molecular Biology* 2002;49:567–77.