New Plant Breeding Techniques
Update of the 2012 Baseline Report

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Impressum

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Author: Benno Vogel, AWEL, SBS.
Translation: John Barrett
Supervision AWEL: Christina Stadler, SBS
Supervision FOEN: Khaoula Belhaj Fragnière, Biotechnology Section
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Summary

Over recent years, several new plant breeding techniques [NPBT] have been developed which require clarification as to how they, and the products derived therefrom, are to be regulated legally. In order to evaluate the need for action arising from NPBT, the Federal Office for the Environment [FOEN] had the Biosafety Section of the Office for Waste, Water, Energy and Air [WWEA] draw up a baseline report in 2012. This present report partially updates that baseline report. It deals with twenty-two NPBT, describing each technique individually, while outlining their potential applications in plant breeding as well as their current state of development.
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1. Introduction

With the latest advances in molecular biology and biotechnology, several new plant breeding techniques [NPBT] have been developed that share a common attribute: While all are based on the use of genetic engineering methods, the resulting end products may nonetheless be free from foreign genes. Some of these NPBT have already been deployed in breeding programmes in the commercial sector. The first NPBT plant-varieties are emerging on the market in North America. The launch of additional NPBT products is to be anticipated in the coming years worldwide.

Given the manifest development of NPBT, it has become increasingly difficult to draw a distinct dividing line between genetic engineering and conventional breeding methods. Hence, uncertainty prevails as to whether the plants derived from the application of NPBT are, seen from a legal perspective, GMOs or not. This uncertainty has triggered an international debate with regard to how NPBT products are to be legally regulated, and how the NPBT governance is to be designed.

In Switzerland, the question remains open as to how NPBT and products derived therefrom are to be regulated. To address this existing legal uncertainty, the Federal Office for the Environment [FOEN] as the leading agency responsible of the Genetic Engineering Act [GTA/GTG] commissioned the Biotechnology Section [SBS] at the Office for Waste, Water, Energy and Air [WWEA] to prepare a baseline report on the NPBT (Vogel 2012). That report deals with the then known NPBT and poses questions which arise in connection with the therein described NPBT on a legal and/or regulatory level.

This report is a partial update of the 2012 baseline report. It covers 22 NPBT, outlining the individual techniques, their potential applications and their current state of development. Seven techniques described herein do not feature in the 2012 report: CRISPR/Cas9 technique (see Section 6); RNAi-induced CMS; Transgene-directed Mutagenesis; TraitUp-Technique; Marker Gene-assisted Crossbreeding; Marker Gene-aided Haploid Breeding; and Developmental Reprogramming.

2. Cisgenesis

Cisgenesis is a novel concept for the transformation of plants. In contrast to transgenesis, in which genes are transferred between x-arbitrary types of organisms, the concept of cisgenesis involves plants being exclusively transformed with native genes, or genes from closely related and sexually compatible species (Holme et al. 2013; Lusser et al. 2012). The transformed genes thereby exhibit their natural orientation; carry their own introns and are flanked by their native promoters and terminators (Holme et al. 2013; Schouten et al. 2006a). Table 1 summarises the key differentiators between Transgenesis and Cisgenesis.

2.1 Description of the Technique

Cisgenesis involves the genome of plants being transformed with one or more cisgenes. Cisgenes are genes from the natural gene pool of the plant to be transformed; that means, they stem either from the species itself, or from a related, sexually compatible plant. Cisgenes are isolated from a plant cell and subsequently inserted unaltered into the genome of another plant cell. Hence, another distinguishing feature of cisgenes is: they exhibit their normal-sense orientation; they have their introns and are flanked by their promoters and terminators (Figure 1).
Cisgenes are inserted in the genome using the same gene transfer methods that are commonly used in the production of GM plants, wherein the transfer of cisgenes mainly takes place at present with the aid of Agrobacterium. Besides established transformation methods, NRE techniques can be used for producing cisgenic plants (see Section 6).

Table 1: Transgenesis and Cisgenesis - Two different Approaches for the Transformation of Plants (as per Molesini et al. 2012).

<table>
<thead>
<tr>
<th>Source of DNA coding sequences</th>
<th>Transgenesis</th>
<th>Cisgenesis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Source of regulatory DNA sequences</td>
<td>All type of organisms</td>
<td>The same species or sexually compatible species</td>
</tr>
<tr>
<td>Type of genetic construct</td>
<td>New combinations of coding and regulatory sequences</td>
<td>Perfect copy of a natural gene</td>
</tr>
<tr>
<td>Orientation of the expressed sequence</td>
<td>Sense or antisense orientation; Hairpin constructs</td>
<td>Sense-orientation</td>
</tr>
<tr>
<td>T-DNA border sequences</td>
<td>Left and right border sequences of Agrobacterium T-DNA</td>
<td>No specific requirements</td>
</tr>
<tr>
<td>Selectable marker gene</td>
<td>Presence of markers</td>
<td>Lack of markers or cisgenic markers</td>
</tr>
</tbody>
</table>

Since cisgenic plants, by definition, should contain no foreign genes, they must be generated in such a way that they either carry a cisgenic marker (Rosellini 2011), or are free of marker genes. Various approaches exist to produce marker-free cisgenic plants, whereby it is possible to remove marker genes from transformed plants, or to directly transform plants without marker genes (Breyer et al. 2014).

![Figure 1: Structure of a typical native gene.](image)

**Figure 1**: Structure of a typical native gene. The gene is part of a chromosome and consists of a promoter and terminator, 5'- and 3'-untranslated regions (UTR), as well as exons and introns. The promoter and terminator are regulatory DNA sequences. During the transcription process, the exons will be fused together and subsequently get translated into a protein. Figure as per Schaar & Visser (2009).
2.1.1 Definition-related Aspects

Cisgenesis is a concept that is defined in different ways in the scientific literature (Holme et al. 2013). Irrespective of these differing definitions, some aspects remain that currently hinder a completely “watertight” interpretation to definitively set out what constitutes cisgenesis (Prins & Kok 2010). The aspects that could lead to a “grey zone” in defining Cisgenesis (Prins & Kok 2010) are hereafter outlined.

**Definition’s Point in Time:** Currently, it remains unclear at what exact point in the process the definition should be applied. Does the definition refer to the intended genetic modification, or merely to the modification that has actually taken place in the plant (Prins & Kok 2010)?

**Codon-Optimisation:** If a cisgenic sequence is known, it is then possible, in principle, to synthetically generate the cisgenes, and hence to adapt the sequence to the codon preferences of the plant which is to be transformed. It remains unclear to what extent synthetic genes with optimised codons fall under the definition of cisgenesis, if at all (Prins & Kok 2010).

**Integration of Partial Cisgenes:** Plants transformed with cisgenes may not only carry complete copies of the cisgenes but also rearrangements and partial copies of the cisgenes. It remains unclear whether plants with rearrangements and/or partial cisgenes could be considered cisgenic (Prins & Kok 2010).

**Source of Cisgenes:** As to the source of the cisgenes, various terms are used in the different definitions of Cisgenesis: Cisgenes can stem from the “natural gene pool”, or from the breeder’s gene pool, or yet again from “crossable species”, or “sexually compatible species”. The following questions need clarification: Can genes, which can be solely introgressed into a plant by bridge crossing, be used as cisgenes? Can genes, which can be solely introgressed into a plant via wide crosses and embryo-rescue techniques, be used as cisgenes? Does the answer to these two questions depend on whether the genes have already being introgressed via bridge crossing, or via wide crosses, and hence present in the genome of a “directly” crossable plant?

**Transfer of Organelle Genes:** In addition to the nucleus cell’s genome, plants also carry organelle genomes of the plastids and the mitochondria. Genes from organelle genomes can also be transferred with established transformation methods. To what extent, and in which cases such genes could be defined as cisgenic remains to be discussed (Prins & Kok 2010). EFSA (2012a) considers the transfer of organelle genes between crossable – sexually compatible – plants as Cisgenesis, if the genes are integrated into the same organelle from which they originate.

**Insertion of “Superfluous” Sequences:** The insertion of superfluous sequences is a consistent feature of current transformation methods, especially with Agrobacterium-based methods. These superfluous sequences may include the following: vector backbone sequences (Gelvin 2003); sequences from *Agrobacterium’s* chromosomal genome (Ülker et al. 2008); T-DNA border sequences (Holme et al. 2012); synthetic sequences (e.g. multiple cloning sites located in T-DNA; Holme et al. 2012) as well as recombinases recognition sequences, which can remain behind in the genome of the plant with the use of certain methods for removing marker genes (with Cre/loxP recombination system, a single *loxP* sequence is left behind; e.g. Terada et al. 2010). In those cases where superfluous sequences are not linked to cisgenes, the sequences can be removed via segregation. Were segregation not possible, the superfluous sequences remain in the genome. Hence, the question may arise in certain cases as to how the occurrence of superfluous sequences needs to be assessed with regard to the definition of Cisgenesis. Since most cases involve short sequences, it is probable that homologous or mostly homologous sequences are to be found in the genome of the transformed plant; hence, the sequences could again be considered as native to the plant.
With regard to superfluous sequences occurring in cisgenic plants, the focus of discussion, to date, in the literature has primarily been the presence of T-DNA border sequences. Moreover, occasionally an explicit distinction is also drawn between “Cisgenesis” and “Cisgenesis with T-DNA border sequences” (e.g. EFSA 2012a; NTWG 2011).

2.2 Potential Applications in Plant Breeding

Cisgenesis is deployed in plant breeding to genetically improve existing varieties. Since the genes used in the process are from the plant’s natural gene pool, Cisgenesis affects the same genetic material, which is also used in conventional crossbreeding. However, compared with crossbreeding, Cisgenesis offers the advantage that the desired genes can be transferred without linkage drag (Eckerstorfer et al. 2014; Lusser et al. 2012; Jacobsen & Schouten 2009). Hence, Cisgenesis can accelerate the breeding process because the lengthy backcrossing required to remove linkage drag is omitted. This time saving is particularly significant for crops that are propagated vegetatively, or have a long generation time (Lusser et al. 2011; Schaart & Visser 2009). Moreover, it could also play a crucial role in cases where resistance genes should be pyramided (Lusser et al. 2011).

Among the traits Cisgenesis could improve in a given plant are: improved disease resistance; altered plant architecture and improved fodder characteristics (Holme et al. 2012; Han et al. 2011; Vanblaere et al. 2011).

2.3 Current State of Development

The concept of Cisgenesis was initially presented in 2000 (Holme et al. 2013), and subsequently became internationally known through the publications of Schouten et al. (2006 a/b). Since then a few cases of cisgenic plants have been described in the literature: apple (Chizzali et al. 2016; Kost et al. 2015; Krens et al. 2015; Würdig et al. 2015; Vanblaere et al. 2014/2011); barley (Holme et al. 2012); and potato (Jo et al. 2012). Among plant-varieties in which the first stage of the development of cisgenic plants have been reported are: citrus plant (An et al. 2013); Chinese cabbage (Konagaya et al. 2013); chestnut (Corredoira et al. 2012); durum wheat (Gadaleta et al. 2008); grapevine (Dhekney et al. 2011); melon (Benjamin et al. 2009); pear (Righetti et al. 2014); and poplar (Han et al. 2011).

Currently in the EU, applications have been submitted for release experiments of at least five cisgenic plants (JRC 2015): scab-resistant apple (Netherlands); fire-blight resistant apple (Switzerland); apple with increased anthcyanin content (Netherlands); phytophthora-resistant potato (Belgium, Netherlands and Switzerland); and barley with improved phytase-activity (Denmark).

3. Intragenesis

Intragenesis is a new concept for the transformation of plants. In contrast to Transgenesis, in which genes are transferred between x-arbitrary organisms, the Intragenesis concept encompasses the principle that plants are transformed only with DNA belonging to the same species or to a cross-compatible species (Table 2). Unlike Cisgenesis (Section 2), with Intragenesis it is possible to recombine the genetic material prior to transformation (Holme et al. 2013; Lusser et al. 2012/2011; Molesini et al. 2012; Rommens et al. 2011/2007; Rommens 2010/2007; Schaart & Visser 2009). Moreover, the transformation vectors used for Intragenesis consist of functional DNA fragments, which are derived from the genome of the species to be modified, or from the
genome of a cross-compatible species (concept of an intragenic vector; Molesini et al. 2012; Rommens et al. 2011/2005; Conner et al. 2007; Rommens 2004).

3.1 Description of the Technique

With Intragenesis, the genome of plants is transformed with one or more intragenes. Intragenes are newly combined genetic constructs whose DNA sequences stem from either the same plant species, or from a related, sexually compatible species. Among possible new combinations are: removal of introns; exchange of promoters and terminators; placing the genetic elements in antisense manner or in a sense-antisense orientation [hairpin constructs] (Molesini et al. 2012).

Intragenes are introduced into the genome of a plant by using the same transformation methods commonly deployed in the generation of GM plants, for example, with the biolistic techniques, or with the aid of *Agrobacterium*. Aside from established transformation methods, NRE technique can also be used for Intragenesis (see Section 6).

Table 2: Transgenesis and Intragenesis - Two different Approaches for the Transformation of Plants (As per Molesini et al. 2012; Rommens et al. 2011).

<table>
<thead>
<tr>
<th>Source of coding DNA-sequences</th>
<th>Transgenesis</th>
<th>Intragenesis</th>
</tr>
</thead>
<tbody>
<tr>
<td>All types of organisms</td>
<td>The same species or sexually compatible species</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Source of regulatory DNA-sequences</th>
<th>Transgenesis</th>
<th>Intragenesis</th>
</tr>
</thead>
<tbody>
<tr>
<td>All types of organisms</td>
<td>The same species or sexually compatible species</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Type of genetic construct</th>
<th>Transgenesis</th>
<th>Intragenesis</th>
</tr>
</thead>
<tbody>
<tr>
<td>New combinations of coding and regulatory sequences</td>
<td>New combinations of coding and regulatory sequences</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Orientation of expressed sequence</th>
<th>Transgenesis</th>
<th>Intragenesis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sense or antisense orientation; hairpin constructs</td>
<td>Sense or antisense orientation; hairpin constructs</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>T-DNA border sequences</th>
<th>Transgenesis</th>
<th>Intragenesis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Left and right borders of <em>Agrobacterium</em> T-DNA</td>
<td>Left and right borders are plant-derived DNA sequences [P-DNA]</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Selectable marker genes</th>
<th>Transgenesis</th>
<th>Intragenesis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Presence of markers</td>
<td>Either lack of markers or presence of plant-derived marker genes</td>
<td></td>
</tr>
</tbody>
</table>

Since intragenic plants, by definition, should contain no foreign sequences, they must be generated in such a way that they either carry an intragenic marker (Rosellini 2011), or are free of marker genes. Various approaches are available to produce marker-free intragenic plants, whereby it is possible to remove marker genes from transformed plants, or to directly transform plants without marker genes (Breyer et al. 2014).

Additional sequences can be inserted into the genome of a plant along with intragenes using current transformation methods. These sequences include, inter alia, T-DNA border-sequences and vector backbone sequences. In order to avoid that sequences foreign to the species thereby end up in the genome of an intragenic plant, researchers have developed the concept of P-DNA, or the concept of intragenic vector (Holme et al. 2013; Rommens 2004; Rommens et al. 2004).
3.1.1 P-DNA Concept

If intragenes are delivered into plant cells with the aid of *Agrobacterium*, the insertion is done via T-DNA [transfer DNA]. This T-DNA is flanked at both ends by the so-called left [LB] and right [RB] border sequences, which are needed for insertion of the intragene. LB and RB are a maximum of 25 base pairs in length (Gelvin 2003). The T-DNA strand inserted into the genome of the plant, normally contains 21-22 nucleotides of the LB and 3 to 4 nucleotides of the RB (EFSA 2012a; Schaart & Visser 2009; Conner et al. 2007), whereby random deletion of nucleotides of the left and the right border can also occur during insertion. Hence, the right border can often be entirely absent in the insertion, and the inserted left border can be shortened to two nucleotides (Prins & Kok 2010). If sequences of the left border and/or the right border are inserted along with the intragene into the genome of a plant, the plant would then carry foreign sequences; for which reason they would no longer fall under the definition of Intragenesis. To circumvent this, P-DNA is used instead of T-DNA in the transformation. P-DNA is functionally identical to T-DNA. However, its left border and right border comprise sequences derived from the plant that is to be transformed, or from a cross-compatible species. In preparing P-DNA, primarily two approaches are pursued: (I) The first approach is to search for sequence regions in the genome of a plant, which are homologous with the left border and right border of *Agrobacterium* to such an extent that they can assume their function. If such sequences are found, they could be used in the P-DNA as the left border and the right border (Rommens et al. 2005/2004). (II) The second approach is to search for sequence regions in the genome, which functionally result in the left border and the right border of the T-DNA, if combined (Conner et al. 2007). The P-DNA is thus constructed *in silico* from plant sequences. In the case of the right border, an additional or third approach is possible: Since normally only the first 3 to 4 nucleotides of the right border are inserted into the genome of the plant, chimeric P-DNAs can be produced, in which the first four nucleotides of the right border originate from plants, while the remainder is identical with the authentic right border of the *Agrobacterium* (Conner et al. 2007).

3.1.2 Intragenic Vector Concept

Since along with the left and right border of the T-DNA, additional sequences from the vector can be inserted into the genome of a plant, intragenic vectors are constructed to minimize the insertion of sequences foreign to the species (Rommens et al. 2011; Conner et al. 2007). The intragenic vector concept consists of identifying sequences in the genome of a plant to be transformed (or in a sexually compatible congener), which are functionally identical to vector backbone sequences. If such sequences are found, they could be used for constructing a vector (Conner et al. 2007).

3.1.3 Definition-related Aspects

Intragenesis is a concept defined in diverse ways in the literature. Among aspects in which the respective definition differs, or is not dealt with, are:

**Codon Alterations**: Since an intragene can be synthetically produced, it is possible to adjust its sequence to the codon preferences of the plant that is to be transformed. It is unclear to what extent synthetic genes with optimised codons are covered by the definition of Intragenesis or not.

**Source of delivered Genetic Material**: Regarding the source of the intragenes, various terms are used in the differing definitions of Intragenesis: The sequences of the intragenic constructs could stem from the “natural gene pool” of a plant, from a “breeder’s gene pool”, or from “crossable species” or from “sexually compatible species”. The following questions require clarification: Can genes, which can only be introgressed into a plant...
via bridge crossing, be used as intragenes? Can genes, which can only be introgressed into a plant via a wide crosses or embryo-rescue techniques, be used as intragenes? Does the answer to the two preceding questions depend upon whether the genes have already been introgressed via bridge crossing or wide-crosses, and, hence present in the genome of a “directly” crossable species?

3.2 Potential Applications in Plant Breeding

Intragenesis can be employed in plant breeding to genetically improve plants. Since the genetic material used can be recombined before transformation, the technique offers various possibilities. Hence, Intragenesis not only allows the expression of new genes, but also the silencing of, the over-expression or modification in tissue activity of endogenous genes. Among traits that could be generated by Intragenesis are: improved disease resistance; reduced allergen content, altered starch composition; increased vitamin- and reduced lignin content (see Rommens 2007).

3.3 Current State of Development

The concept of Intragenesis was initially formulated in 2004 (Rommens 2004; Rommens et al. 2004). Since then, P-DNAs for various crops have been developed (Holme et al. 2013; Rommens et al. 2011). The development of intragenic plants has been pursued in the following crops: apple (Joshi et al. 2011); alfalfa (Weeks et al. 2008); citrus plant (An et al. 2013); grapevine (Espinoza et al. 2013); pasture grass (Puthigae et al. 2010); poplar (Lu et al. 2015); potato (Brummel et al. 2015; Chawla et al. 2012; Rommens et al; 2008/2006/2004); and strawberry (Schaart 2004).

To date in the EU, requests have been submitted for the experimental release for intragenic apple and potato plants (JRC 2015; Lusser et al. 2011).

In the USA, the launching by the Simplot Company of Innate potatoes has resulted in the first intragenic plants being available on the market (Halterman et al. 2016; Waltz 2015). The first generation Innate potatoes, whose cultivation was authorised in 2014, possess lower levels of asparagine and do not turn brown (or are less susceptible to black spot). In 2015, the area under cultivation was approximately 160 hectares and is due to be increased to 800 hectares in 2016 (Transgen 2015.) In 2015, the second generation Innate potato was approved for cultivation. Compared with the first generation, it moreover exhibits a resistance to Phytophtora infestans and is scheduled to appear on the US market in 2017.

4. Grafting on GM Rootstock

Grafting is an old technique, essentially consisting of a shoot (scion) of one particular plant being merged with a stock (rootstock) of another plant. If grafting techniques are combined with genetic engineering methods, this can be done in three distinct ways (Figure 2): (I) grafting a non-GM scion onto a GM rootstock; (II) grafting a GM scion onto a non-GM rootstock, and (III) grafting a GM scion onto a GM rootstock.

Cases of (I) and (II) are also referred to as transgrafting in the scientific literature (Albacete et al. 2015; Song et al. 2015; Eckerstorfer et al. 2014; Lemgo et al. 2013; Haroldsen et al. 2012).

The descriptions in the following sections deal exclusively with case (I). This method is regularly used in plant breeding and relevant to the present study, because, from a regulatory perspective, the question arises as to how the offspring and the products are to be handled.
4.1 Description of the Technique

The method combines traditional grafting technique with genetic engineering. First, genetically engineered rootstocks are produced. This happens by transforming a plant through the use of classical genetic engineering methods (e.g. by means of Agrobacterium, or with biolistics). The rootstocks are then isolated, or obtained from the transformed plant. Finally, the scions are grafted onto the GM rootstocks (Lusser et al. 2011).

Transgenes, cisgenes, intragenes as well as other genetic constructs can be deployed for the genetic modification of the rootstock (Song et al. 2015; Lusser & Davies 2013).

4.2 Potential Applications in Plant Breeding

The conventional grafting method is mainly employed for ornamental and fruit trees, vegetables and flowers. Chimeras of non-GM scions and GM rootstocks can be produced in all graftable species transformed by means of genetic engineering methods.

Grafting non-GM scions onto GM rootstocks may be applied in plant breeding mainly for the three following objectives (Lusser et al. 2011; Schaart & Visser of 2009):

1. **Use of Rootstocks with new Traits**: Rootstocks will be genetically engineered in such a way that they are resistant to soil-borne diseases, or will posses improved rooting ability (e.g. Geier et al. 2008; Park et al. 2005; Escobar et al. 2002). Both of these modifications could increase the yield of products harvested from the scions.

2. **Changes in the Scion’s Traits**: Rootstocks are genetically engineered in such a way that they form proteins or siRNAs, which are deliberately transported into the scions, hence changing the scion’s traits in a desirable manner (Song et al. 2015; Zhao & Song 2014; Kasai et al. 2011; Notaguchi et al. 2008; Dutt et al. 2007). In this way, new traits can be introduced in a series of genetically distinct scions without that these need to be genetically engineered (Lusser et al. 2011).

3. **Tool for other Breeding Techniques**: The rootstocks are modified in such a way that they form proteins or siRNAs, which are transported into the scions. However, the objective thereby is not to directly induce commercial traits in the scions but rather to enable breeding techniques such as Early Flowering (Febres et al. 2011; Zhang et al. 2010a; see Section 15); Reverse Breeding (Dirks et al. 2009; see Section 18), or RdDM (Bai et al. 2011; see Section 13).

In addition to these three intended applications, the use of rootstock with transformed plastids in plant breeding is also recently being discussed (Wani et al. 2015; Bock 2014; Thyssen et al. 2012; Stegemann et al. 2012).
Since chloroplasts can be transported from the rootstock into the scion, transplastomic rootstocks can be deployed to introduce transformed plastids into different varieties (Thyssen et al. 2012), or to breed transplastomic varieties in those plant species whose plastids cannot be transformed (Stegemann et al. 2012). Since use of these applications will result in transplastomic plants, and hence GMOs, they are not discussed in the following.

4.3 Current State of Development

The earliest description of the concept for the use of GM rootstocks was in 1991 (MacKenzie et al. 1991). Since then the technique has been tested on numerous crops. Testing on genetically engineered rootstock has been carried out in the main, though not exclusively, on tree species such as: apple (Smolka et al. 2010; Xu et al. 2009; Zhu et al. 2001); cherry (Zhao & Song 2014; Song et al. 2013); grapevine (Krastanova et al. 2010; Hemmer et al. 2009; Aguero et al. 2005; Gambino et al. 2005; Geier et al. 2008; Vigne et al. 2004; Coutos-Thevenot et al. 2001); orange (La Malfa et al. 2009); plum (Nagel et al. 2010); poplar (Wang et al. 2012); walnut (Vahdati et al. 2002); and watermelon (Kim et al. 2015a; Han et al. 2015/2009; Youk et al. 2009; Park et al. 2005). Moreover, the use of genetically engineered rootstocks has been investigated in cucumber, potato, pea, tobacco and tomato (review by Lusser et al. 2011). In some instances, genetically engineered rootstocks have already been tested in field experiments. In the EU, for example, corresponding applications have been submitted for apple, citrange, grapevine, pear, plum and orange (JRC 2015).

5. TraitUp-Technique

The TraitUp-Technique uses recombinant geminivirus-based plasmids to produce plants with novel traits. The plasmids are thus introduced into the plants, where they automatically replicate, but do not integrate into the genome. Given that the plasmids cannot be inherited, the offspring of the treated plants may be free from recombinant DNA (Morflora 2014).

5.1 Description of the Technique

The TraitUp-Technique is based upon the use of recombinant IL-60- or p1470-plasmids (Gover et al. 2014; Mozes-Koch et al. 2012; Peretz et al. 2007). Both plasmids are “disarmed” forms of tomato yellow leaf curl virus [TYLCV], and are constructed in such a way that they can spread themselves within a plant and replicate themselves autonomously, without being inserted into the plant genome. The plasmids can be directly delivered into seeds from plants (Lapidot et al. 2014). Given that the plasmids can be equipped with expression-cassettes or RNAi-constructs, plants expressing foreign genes or exhibiting silenced genes can be produced (Mozes-Koch et al. 2012; Peretz et al. 2007). Based on the current state of knowledge, the plasmids are not inherited.

5.2 Potential Applications in Plant Breeding

Through the application of the TraitUp-Technique similar objectives can be pursued as with Trans-, Cis- or Intragenesis. Unlike these transformation-based techniques, however, the desirable traits will not be generated during a protracted breeding process, but rather through the treatment of the seed with recombinant plasmids.
5.3 Current State of Development
TraitUp-plasmids are thought to be expressible in more than forty different crop species (Dietz 2013), including banana, barley, carrot, corn, olive, pepper, soy, tomato and wheat (Peretz et al. 2007). Currently, only few reports on the application of TraitUp Technique can be found. In tomato, the technique has been used to express a complete bacterial operon, which resulted in plants producing the anti-fungal antibiotic pyrrolnitrin (Mozes-Koch et al. 2012). Furthermore, the technique is being tested for the control of Fusarium crown rot on tomatoes (Braverman 2013). In apple, TraitUp-plasmids are currently being used to develop scab-resistant varieties (Cusin et al. 2014).

6. NRE Technique
The NRE technique [NRE = new restriction enzymes] is a new breeding technique, in which site-specific nucleases are employed to modify the genome of plants (Lee et al. 2016; Weeks et al. 2016; Baltes & Voytas 2015; Kim et al. 2015b; Osakabe & Osakabe 2015a/b; Rinaldo & Ayliffe 2015; Xiong et al. 2015; Fichtner et al. 2014; Chen & Gao 2014; Kathiria & Eudes 2014; Nakayama et al. 2014; Pauwels et al. 2014; Puchta & Fauser 2014; Voytas & Gao 2014; Podevin et al. 2013; Voytas 2013; Curtin et al. 2012; Tzfira et al. 2012). Genetic engineering methods are employed during the process in order to introduce genes encoding site-specific nucleases into plant cells. Since these genes are no longer needed in the final product, transgene free plants can be obtained from use of the NRE technique (e.g. Wolt et al. 2016; Voytas & Gao 2014; Pauwels et al. 2014).

Various appellations are ascribed to the NRE technique in the literature. These include: genome editing (Sprink et al. 2015; Kathiria & Eudes 2014.); genome editing with engineered nucleases [GEEN] (Osakabe & Osakabe 2015a/b); precision genetic engineering [PGE] (Nakayama et al. 2014); precision genetic modification [PGM]-techniques (Fichtner et al 2014); targeted genome modification [TagMo]-technologies (Kokotovich & Kuzma 2014); nuclease-based gene targeting [NBGT] (Pauwels et al. 2014); site-directed nuclease [SDN]-techniques (Lusser & Davies 2013); site-specific nuclease [SSN]-techniques (Chen & Gao 2014); and nuclease-mediated site-directed mutagenesis (Eckerstorfer et al. 2014).

6.1 Description of the Technique
The NRE technique is based upon the use of site-specific nucleases and utilising the plant cell’s own DNA repair processes. The site-specific nucleases are used to generate double-strand breaks at predetermined genomic loci of the plant. The plant’s own repair processes are initiated where the nuclease creates a double-stranded cut. The double-strand break repair is carried out either by non-homologous end joining [NHEJ] or, if a DNA molecule with homologies to the sequence flanking the cleavage site is available, through homologous recombination [HR]. At the cleavage site the NHEJ leads to substitution, insertion or deletion of nucleotides. The HR, in turn, results in gene replacement, or the insertion of new genes at the cleavage site.

The following describes which types of nucleases can be used with the NRE technique; which categories of modifications can be generated using nucleases, and what methods are available to deliver the nucleases into plant cells.
6.1.1 Types of Nucleases

Currently, four types of site-specific nucleases are generally available for the NRE technique (Weeks et al. 2016; Baltes & Voytas 2015; Rinaldo & Ayliffe 2015; Puchta & Fauser, 2014): Meganucleases [MN], zinc finger nuclease [ZFN], TALE nucleases [TALEN] and CRISPR/Cas9. Moreover, RNA-guided FokI nucleases and nickase have also been tested (Lee et al. 2016).

Meganucleases [MN]: MN are naturally occurring endonucleases that generally recognise specific DNA segments between 12 and 40 base pairs. MN can be modified so as to adapt to their recognition sequences (Rinaldo & Ayliffe 2015).

Zinc Finger Nucleases [ZFN]: ZFN are synthetically produced nucleases, consisting of a DNA binding domain of zinc fingers and the nuclease domain of the FokI restriction enzyme. Essentially, ZFN are so designed that they are capable of recognising a specific 9 to 18 base-pair DNA segment. Since the FokI activity requires dimerization, it invariably requires a pair of ZFN to be able to generate a double-strand break at a predetermined genomic site (Lee et al. 2016).

TALE nucleases [TALEN]: Just as with ZFN, TALEN are synthetically produced nucleases. They consist of the DNA binding domain from transcription activator-like effector [TALE] proteins and the nuclease domain of the FokI restriction enzyme. TALEN can be designed in such a way that they recognize a specific DNA segment of 30 to 40 base-pair in length (Kim & Kim 2014). Given FokI activity requires dimerization, it invariably needs, just as with the ZFN, a pair of TALEN to obtain a functional nuclease.

CRISPR/Cas9: The CRISPR/Cas9 system comprises two components: the Cas9 nuclease and a so-called guide RNA [gRNA]. The gRNA directs the Cas9 nuclease to a predetermined genomic site where it generates a double-strand break. As a DNA-specific recognition part, the gRNA can be so designed that it is capable of recognising a roughly 20 base-pair DNA segment (Belhaj et al. 2015).

RNA-guided FokI nucleases: An RNA-guided FokI nuclease is a synthetically made fusion product from the nuclease domain of the FokI restriction enzyme and a catalytically inactive Cas9 nuclease [denoted dCas9]. Just as with CRISPR/Cas9, the DNA recognition is achieved by a gRNA. Since FokI activity requires dimerization, it invariably takes a pair of RNA-guided Fok nucleases to be able to produce a double-strand break at a predetermined genomic site (Lee et al. 2016; Bortesi & Fischer 2015).

Nickases: Nickases are synthetically produced enzymes that generate single-strand breaks at predetermined genomic loci. They can be developed from ZFN, TALEN and CRISPR/Cas9, and should enable homologous recombination to be induced at the cleavage site, without thereby activating the NHEJ (Lee et al. 2016; Kim & Kim 2014).

6.1.2 Categorisation of Producible Modifications

With the use of NRE techniques, a wide variety of modifications can be produced in the plant genome. So as to both simplify their description and facilitate a discussion of their regulatory oversight, a classification into three distinct categories of the possible modifications has been proposed in the literature (Podevin et al. 2013; Lusser et al. 2011). While this categorisation schema is broadly followed herein, a fourth category will be added (Figure 3). The so-called multiplex genome editing is not, however, directly attributable to any of the four-mentioned categories. It describes applications of the NRE technique in which two or more different predetermined genomic loci are simultaneously modified.
**NRE-1:** This category designates the application of the NRE technique whereby non-specific mutations are generated at a predetermined genomic site. The nucleases are delivered into plant cells without a repair template. Within the cells, the nucleases bind to the predetermined site, where they produce a double-strand break. Repair of the cleavage site takes place by means of NHEJ, whereby indels are generated. Applying the NRE-1 technique enables the knocking out of targeted genes (Gene-Knockout; Podevin et al 2013; Lusser et al 2011).

**NRE-2:** This category designates the application of the NRE technique whereby specific mutations are generated at a predetermined genomic site. In addition to the nucleases, repair templates are also thereby introduced into plant cells. The templates can be double-stranded DNA vectors, single-stranded DNA oligonucleotides (e.g. Svitashev et al. 2015), or chimeric RNA/DNA oligonucleotides (Wang et al 2015a.) and exhibit – aside from the nucleotides to be exchanged – homologies to the chromosomal sequence, in which the nucleases introduce a double-strand break. Repair of the cleavage site is achieved through homologous recombination between the broken genomic sequences and the introduced template. Applying the NRE-2 technique enables the replacement of genes, the correction of unwanted spontaneous mutations or the targeted generation of new mutations (Podevin et al. 2013; Lusser et al. 2011).

**NRE-3:** This category designates the application of the NRE technique whereby genes are inserted at a predetermined genomic site. The nucleases are introduced jointly with a donor DNA into plant cells. The donor DNA contains the sequence to be inserted, as well as flanking sequences that exhibit homologies to the targeted site. The insertion of the donor DNA sequence is achieved by means of homologous recombination. Through the application of the NRE-3 technique cisgenes, intragenes, transgenes or RNAi constructs can be integrated into the target sequence of the genome. Simultaneously, it is possible to knockout targeted plant endogenous genes (Podevin et al. 2013; Kim & Kim 2011; Lusser et al. 2011). Plants derived from application of NRE-3 technique are deemed as GMOs under current laws.

**NRE-4:** This category designates the application of the NRE technique whereby chromosomal deletions, inversions, duplications or translocations are generated. With the help of nucleases, double-strand breaks are generated at two predetermined genomic sites. Should the breaks lie on the same chromosome, it may lead to

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**Figure 3:** A simplified schematic presentation of the NRE variants 1-4. Inversions, duplications and translocations are not herein presented. Refer to the body-text for a more comprehensive description of the four variants.
deletion or inversion of the sequence between the breaks. Should, however, the double-strand breaks lie on different chromosomes, translocations may result (Baltes & Voytas 2015; Puchta & Fauser 2014; Podevin et al. 2013). As with the application of NRE-1, repair of the double-strand breaks occurs invariably through the use of NHEJ.

6.1.3 Methods for Delivering Nucleases

In order to be able to produce the described modifications with the site-specific nucleases, these nucleases and, where applicable, additional reagents such as gRNA, donor DNA or repair templates must be delivered into plant cells. Currently, the introduction of nucleases is mostly achieved in the form of nucleic acids. Moreover, it is possible to deliver nucleases into the cells in the form of proteins (Woo et al. 2015; Luo et al. 2015; Martin-Ortigosa et al. 2014).

In order to deliver genes coding for site-specific nucleases, and for other reagents such as gRNA, repair templates or donor DNA into plant cells, four genetic engineering approaches are available: Transformation, Transient Transfection, VAGE (Section 22) and Agroinfiltration (Voytas & Gao 2014; Tzfira et al. 2012; Mahfouz & Li 2011).

**Transformation:** The nuclease-encoding genes are inserted into the genome of plants with transformation methods. This results in a stable or induced expression in the transformed plants and the nucleases create a double-strand break at a predetermined genomic site. At the end of the process, the nuclease-encoding genes can be removed by means of segregation, resulting in plants that are transgene free (Voytas & Gao 2014).

**Transient Transfection:** Plasmids with nuclease-encoding genes are delivered into protoplasts or into cell suspensions. There, the nuclease-encoding genes are expressed transiently before the plasmid is degraded (Voytas & Gao 2014). Transgene free plants can be regenerated from the transfected protoplasts.

**VAGE:** The nuclease-encoding genes are delivered into plants using viral vectors (VAGE; Section 22) (Ali et al. 2015a/b; Honig et al. 2015; Baltes et al. 2014; Vainstein et al. 2011; Marton et al. 2010). The recombinant virus propagates itself in the infiltrated plant, the nuclease-encoding genes are expressed and the nucleases create a double-strand break at a predetermined genomic site. Depending on the vector, virus-free progeny can be obtained in three ways (Vainstein et al. 2011): (I) regeneration of mutant plant tissue and the potential elimination of the virus by meristem-culture techniques; (II) vegetative propagation of mutated shoots and the potential elimination of the viruses by meristem-culture techniques; (III) obtaining virus-free seeds, if mutated shoots produce flowers and the introduced virus is not transmitted via seeds.

**Agroinfiltration:** The nuclease-encoding genes are temporarily delivered into plant with the aid of Agrobacterium (Mahfouz & Li 2011; see Section 14). This results in gene expression taking place in the infiltrated plants and the nucleases create a double-strand break at a predetermined genomic site. Cells of the infiltrated plant tissue are regenerated into plants. Given agroinfiltration can proceed without insertion of the delivered sequences, regenerated plants, which are free of transgenes, can be obtained.

6.2 Potential Applications in Plant Breeding

The NRE technique, and the CRISPR/Cas9 system in particular, is considered a particularly promising new technique for plant breeding (e.g. Baltes & Voytas 2015; Belhaj et al. 2015). Through its application, achieving numerous breeding objectives is conceivable because it has made it possible to knockout targeted genes in the genome of plants; to specifically correct genes or to add new genes at a predetermined site. Among the breeding
objectives envisaged are: herbicide tolerance; resistance to diseases and pests; increased yield; prolonged shelf life; improvement in nutritional value and optimisation of oil and starch composition (Podevin et al. 2013).

6.3 Current State of Development

Though a relatively new plant breeding technique, the NRE technique has already been tested on numerous crops (refer to summaries in Weeks et al. 2016; Baltes & Voytas 2015). In the vast majority of cases, endogenous plant genes have been specifically knocked out (NRE-1). The feasibility of gene replacement (NRE-2), gene insertion and gene stacking (NRE-3) as well as gene deletion (NRE-4) has also been demonstrated sporadically on crop plants. Moreover, with CRISPR/Cas9 multiplex genome editing has been achieved. The following outlines on which crops MN, ZFN, TALEN and CRISPR/Cas9 have already been applied.

MN: To date, scientific reports on the applications of MN on crop species are available for maize (Djukanovic et al. 2013; Gao et al. of 2010) and cotton (D’Halluin et al. 2013). The scarcity of publications on the subject – particularly if compared with ZFN and TALEN – can be likely explained by the fact that a great deal of effort is required to modify the DNA binding domain of MN so that a binding at the desirable site in the genome occurs. Given this lack of flexibility, it is then assumed that MN will not play a decisive role in plant breeding (Puchta & Fauser 2014). A review of the applications of MN in plants is to be found in Daboussi et al. (2015).

ZF:N: In 2005, the earliest demonstration of the targeted modification of the plant genome with ZFN was achieved in the model plants Arabidopsis and tobacco (Lloyd et al. 2005; Wright et al. 2005). Since then ZFN has been successfully used on numerous crop species – for example, apple (Peer et al. 2015); corn (Ainley et al. 2013; Shukla et al. 2009); fig (Peer et al. 2015); petunia (Marton et al. 2010); rice (Cantos et al. 2014); and soybean (Curtin et al. 2011). Reviews of the applications of ZFN in plants can be found in Petolino (2015); Petolino et al. (2015); and Qi (2015).

TALEN: The first reports on the application of TALEN on plants date from 2011 (Cermak et al. 2011; Mahfouz et al. 2011). Since then TALEN have been applied on several crop species including, barley (Gurushidze et al. 2014; Wendt et al. 2013); cabbage (Sun et al. 2013); corn (Char et al. 2015; Liang et al. 2014); potato (Clasen et al. 2016; Nicolia et al. 2015); rice (Zhang et al. 2016; Ma et al. 2015a; Shan et al. 2015/2013a; Wang et al. 2015a; Chen et al. 2014; Li et al. 2012); soybean (Du et al. 2016; Haun et al. 2014); tobacco (Zhang et al. 2013; Mahfouz et al. 2011); tomato (Cermak et al. 2015; Lor et al. 2014); and wheat (Wang et al. 2014). A review of the previous applications of TALEN on plants can be found in Sprink et al. (2015).

CRISPR/Cas9: The earliest reports demonstrating that CRISPR/Cas9 can be applied to plants were published in 2013 (Belhaj et al. 2013; Feng et al. 2013; Jiang et al. 2013; Li et al. 2013; Mao et al. 2013; Miao et al. 2013; Nekrasov et al. 2013; Shan et al. 2013B; Xie & Yang 2013). Since then, applications of CRISPR/Cas9 in plants have been demonstrated in numerous other studies. Among the crops on which CRISPR/Cas9 has been already been tested are: barley (Lawrenson et al. 2015); cabbage (Lawrenson et al. 2015); maize (Feng et al. 2016; Svitashev et al. 2015; Liang et al. 2014; Xing et al. 2014a); orange (Jia & Wang 2014), poplar (Fan et al. 2015), potato (Wang et al. 2015b); rice (Endo et al. 2015a; Lowder et al. 2015; Ma et al. 2015b; Mikami et al. 2015; Xie et al. 2015; Xu et al. 2015/2014; Zhang et al. 2014; Feng et al. 2013; Jiang et al. 2013; Mao et al. 2013; Shan et al. 2013B; Xie & Jang, 2013); sorghum (Jiang et al. 2013); soybean (Du et al. 2016; Cai et al. 2015; Jacobs et al. 2015; Li et al. 2015; Sun et al. 2015; Michno et al. 2015); tobacco (Gao et al. 2015); tomato (Cermak et al. 2015; Ito et al. 2015; Brooks et al. 2014; Ron et al. 2014); and wheat (Wang et al. 2014; Shan et al. 2013b;
7. Oligonucleotide-Directed Mutagenesis [ODM]

Oligonucleotide-directed Mutagenesis [ODM] is a breeding technique, in which chemically synthesised oligonucleotides are introduced into living cells so as to induce targeted mutations at predetermined sites in the plant genome (Sauer et al. 2016; Gocal et al. 2015; Lusser et al. 2011/2012; Breyer et al. 2009; Iida & Terada 2005; Oh & May 2001). ODM is a generic term, which stands for various approaches and applications. In scientific literature, a number of different names are used for ODM (Lusser et al. 2011; Breyer et al. 2009).

7.1 Description of the Technique

Through the use of ODM, site-specific nucleotide substitutions, insertions or deletions can be induced in the genome of plants (Lusser et al. 2011). Delivering oligonucleotides into plant cells triggers the mutations. These oligonucleotides are chemically synthesised, and exhibit – with the sole exception of the nucleotides to be exchanged – homologies to the target sequence. It is presumed that the mutations occur as a result of the cell’s own gene repair mechanism (Rivera-Torres & Kmiec 2016; Lusser et al. 2011; COGEM 2010; Connor 2010; Iida & Terada 2005). As for the fate of the delivered oligonucleotides, it is anticipated that they do not integrate into the genome, but instead are degraded within the cell. The oligonucleotides used in ODM are approximately 20 to 100 nucleotides long (Lusser et al. 2011; Connor 2010). Two types of oligonucleotides are mainly used for the mutagenesis of plant cells: RNA-DNA oligonucleotides (RDO, Figure 4), or single-stranded DNA oligonucleotides (Sauer et al. 2016). Other types that could also be used include: RNA oligonucleotides; triple helix-forming oligonucleotides [TFO] as well as oligonucleotides with chemically modified nucleic acids such as peptide nucleic acids [PNA], or the so-called locked nucleic acids [LNA] (COGEM 2010; Connor 2010; Breyer et al. 2009).

Various methods enable the delivery of oligonucleotides to plant cells, notably biolistic methods, electroporation, PEG-mediated transfection, micro-injection and lipofection (micro-encapsulation). The choice of method depends, inter alia, upon which cells the oligonucleotides are to be introduced. As a rule, electroporation and biolistic methods are used in plants (Sauer et al. 2016; Gocal et al. 2015). Irrespective of the choice of method used, delivering oligonucleotides to plant cells occurs without vector systems.

7.2 Potential Applications in Plant Breeding

Given that specific mutations in gene sequences or other DNA sequences of a plant can be induced with ODM, the following alterations may be made: modification of a protein’s amino acid sequence; deactivation of a gene (through the introduction of stop codons or reading frame shifts); or change in gene expression (through mutations in the promoter sequence). In other words: using ODM, undesirable genes can be silenced in the plant; useful genes can be activated or efficient proteins/enzymes can be produced.
The possibilities offered by applying ODM are comparable to traditional forms of mutagenesis, whereby ODM has the significant advantage of being able to induce site-specific mutations. Hence, compared with traditional mutagenesis, fewer unintended changes occur in the genome during ODM (ACRE 2011, Lusser et al. 2011; COGEM 2010; Breyer et al. 2009; BAC 2007).

Among the traits which could be bred in plants through the use of ODM are, inter alia: herbicide tolerance; pest and disease resistance; tolerance to abiotic stress; extended durability [shelf-life] as well as modified starch and oil composition (Lusser et al. 2011; Breyer et al. 2009).

*Figure 4*: Simplified schematic representation of ODM-technique for the case in which RNA-DNA oligonucleotides (RDOs) are delivered into protoplasts. Colour code for the RDO: green = DNA sequences which are homologous to the target gene; light green = RNA sequences which are homologous to the target gene; red = mismatch; orange = loops consisting of thymidine residues and "GC clamps". Adapted from Hohn & Puchta (1999).

### 7.3 Current State of Development

Although the first studies describing successful applications of ODM in plants date back to 1999 (Beetham et al. 1999; Zhu et al. 1999), the number of published reports on ODM derived crops plants remains limited. ODM applications in crops have been reported for *banana* (Rice et al. 2000); *maize* (Zhu et al. 1999/2000; Rice et al. 2007).
oilseed rape (Gocal et al. 2015; Ruiter et al. 2003; Gamper et al. 2000); rice (Okuzaki & Toriyama 2004), tobacco (Kochevenko & Willmitzer 2003); and wheat (Dong et al. 2006).

In North America, application of the ODM technique has resulted in a first product for commercial use. Since 2015 in the USA, the US-based company Cibus has produced the oilseed rape 715, which is tolerant to sulphonylurea herbicides, available. Cibus oilseed rape 5715 is scheduled to be on the market in Canada as of 2017 (Cibus 2015). Moreover, a market launch in Europe is being planned (Wolt et al. 2016).

Cibus intends to introduce additional ODM varieties on the market by the end of 2020: a phytophthora-resistant potato as well as herbicide-tolerant varieties of flax and rice (Cibus 2015).

8. T-DNA-mediated Mutagenesis

T-DNA-mediated Mutagenesis is a technique deployed for the targeted mutagenesis of plants (Endo & Toki 2013). If the technique is applied without exogenous positive selection-markers, it will be designated as a “clean” transformation technique, which will result in plants comparable to plants derived from conventional mutation breeding (Saika & Toki 2011).

8.1 Description of the Technique

The T-DNA-mediated Mutagenesis technique enables the targeted insertion, deletion or exchange of one or more nucleotides at a designated sequence of the genome (Endo & Toki 2013). To induce the mutations, first a DNA sequence homologous to the target sequence – apart from the nucleotides to be replaced – is cloned in a T-DNA. The vector with the T-DNA is then brought in Agrobacterium and introduced into plant cells, where it can finally result in a homologous recombination between the delivered sequence and the endogenous target sequence (Saika et al. 2011; Endo et al. 2007). The transformed cells can be regenerated into plants, whereby those are plants selected which exhibit exclusively homologous recombinations. Regenerated plants, in which random T-DNA insertions and/or undesirable ectopic recombinations occurred in addition to homologous recombination, will be discarded.

Targeted Mutagenesis with T-DNA is comparable to the NRE-2 technique (Section 6.1.2). In contrast, however, to the NRE-2 technique, nucleases are not employed.

8.2 Potential Applications in Plant Breeding

T-DNA-mediated Mutagenesis could enable similar applications in plant breeding to those possible with ODM (Section 7) or NRE-2 technique (Section 6).

8.3 Current State of Development

To date, reports on the successful application of the technique have only been published for rice (Endo et al. 2015b/2007; Saika et al. 2011; Saika & Toki 2011).

9. RNAi-induced CMS

With the application of RNAi-induced CMS [cytoplasmic male sterility], the expression of the Msh1-gene is suppressed in plants by means of genetic engineering methods, in order to generate rearrangements in the ...
mitochondrial genome that will result in cytoplasmic-male sterility (Sandhu et al. 2007). The resulting male sterile plants may be transgene free (Sandhu et al. 2007).

9.1 Description of the Technique
As with Developmental Reprogramming (Section 11), a GM plant is produced in a first step, in which the expression of the *Msh1* gene is suppressed by RNAi. Since the *Msh1* protein ensures the stability of the mitochondrial genome, silencing the *Msh1* gene results in rearrangements of the mitochondrial genome in the GM plant. These rearrangements, in turn, may cause male sterility in the plants. If the GM plants are crossed with the untransformed parental plant, the induced CMS will also be retained in the offspring, which no longer exhibit the RNAi construct (Sandhu et al. 2007).

9.2 Potential Applications in Plant breeding
The technique enables the generation of new CMS lines, which can be employed in the production of hybrid seed (Sandhu et al. 2007).

9.3 Current State of Development
To date published reports for generating CMS lines by the use of RNAi-induced silencing of the *Msh1* gene are to be found for tobacco and tomato (Sandhu et al. 2007). Moreover, successful generation of CMS-lines have been reported for millet, sorghum and soybean (Arrieta-Montiel & Mackenzie 2011).

10. Transgene-directed Mutagenesis
Transgene-directed Mutagenesis is based upon employing genetic engineering methods to disrupt the DNA-repair system in plant cells in order to thereby generate mutations in the genome of plants cells (van Marcke & Angenon 2013; Xu et al. 2012a; Chao et al. 2005). Since the genetically engineered modification is no longer needed in the final product, transgene free plants can be obtained (Xu et al. 2012a).

10.1 Description of the Technique
With the application of Transgene-directed Mutagenesis, plants are genetically engineered with expression cassettes or RNAi constructs in such a way that their DNA-mismatch repair system no longer functions properly. Disturbing the DNA repair system results in point mutations (substitutions) in coding regions of the plant genome, as well as in short insertions and deletions in microsatellites (Xu et al. 2012a). These genetic modifications are stably inherited and hence can also be present in the offspring of GM plants, which no longer harbour the expression cassettes and/or the RNAi constructs (Xu et al. 2012a).

10.2 Potential Applications in Plant Breeding
The potential applications of the Transgene-directed Mutagenesis are comparable to those of conventional mutagenesis.

10.3 Current State of Development
In 2005, the technique was initially developed on *Arabidopsis* (Chao et al. 2005) and has subsequently been tested on *rice* (Xu et al. 2012a); *tobacco* (van Marcke & Angenon 2013); and *tomato* (Tam et al. 2011).
11. Developmental Reprogramming

Developmental Reprogramming [DR] is here designated as meaning a technique that uses RNAi to suppress the expression of the Msh1 gene in plants, and hence generates epigenetic modifications that can lead to yield increases (Yang et al. 2015; Santa Maria et al. 2014). Since epigenetic modifications can also be retained in offspring without RNAi-con structs, transgene free plants can be produced (Yang et al. 2015; Santa Maria et al. 2014).

11.1 Description of the Technique

The first step of the technique is to produce GM plants in which the formation of the plant's own Msh1 protein is suppressed by RNAi. This suppression triggers a reaction in the chloroplasts of the GM plants that results in epigenetic modifications of the nuclear genome, and – thereby linked – to a developmental reprogramming with a series of new phenotypic traits (Xu et al. 2012b). These new traits are retained in the offspring of the GM plants independent of the RNAi transgene. Should reprogrammed plants without RNAi constructs be crossed with untransformed parental plants, it can result in offspring with better growth and that deliver higher yields (Virdi et al. 2015; Yang et al. 2015; Santamaria et al. 2014).

11.2 Potential Applications in Plant Breeding

DR-technique might be of interest in breeding because it opens up the possibility to develop plant varieties that deliver higher yields, as with hybrid breeding (Yang et al. 2015; Santamaria et al. 2014).

11.3 Current State of Development

DR-technique is currently being tested on millet, sorghum, soybean and tomato (Yang et al. 2015; Santamaria et al. 2014; Xu et al. 2012b; Mackenzie 2011).

12. RNAi- induced Hypomethylation

The RNAi-induced Hypomethylation technique employs genetic engineering methods to downregulate DNA methyltransferases, or other regulators of DNA methylation in plants, and thereby enables the generation of epiallelic variations (King et al. 2010). Since the genetically engineered modification is no longer needed in the end product, transgene free plants can be obtained.

12.1 Description of the Technique

RNAi-induced Hypomethylation is based on the transient silencing of genes that encode DNA methyltransferase or other DNA methylation regulators such as the chromatin-remodelling factor DDM1 (King et al. 2010). By silencing such genes in plants, the methylation pattern varies at random sites in the genome. Since the modified pattern can be passed onto the progeny, phenotypes with the desirable epialleles can be selected. The gene silencing can be carried out by means of stably transforming RNAi constructs, or through the use of VIGS (Section 21). If the stable transformation approach is applied, plants no longer carrying the inserted RNAi construct can be produced via segregation. Should VIGS be applied, progeny without RNAi-constructs can arise, if a viral vector not transmitted via seeds is used (Section 21).
12.2 Potential Applications in Plant Breeding
With RNAi-induced Hypomethylation, so-called epialleles can be generated in selected plants – these are alleles that have an identical DNA sequence, but are expressed differently. Since epigenetic diversity affects phenotypic diversity, and hence also the plants’ performance, this technique could be of interest in plant breeding.

12.3 Current State of Development
The fact that epiallelic variation can be generated in plants by using RNAi-induced Hypomethylation has been demonstrated in turnip rape (Fujimoto et al. 2008); rice (Higo et al. 2012); and poplar (Zhu et al. 2013).

13. RNA-directed DNA-Methylation (RdDM)
RNA-directed DNA-methylation [RdDM] refers to a plant breeding technique, in which genetic engineering methods are used to methylate targeted genomic regions, and thus to modify the expression of endogenous genes (Kasai & Harada 2015; Kasai & Kanazawa 2013). Since the genetic engineering methods are necessary only for triggering methylation, but are not mandatory for conserving the new methylation pattern, transgene plants can be developed with RdDM (Kasai & Harada 2015; Kasai & Kanazawa 2013; Lusser et al. 2012/2011; Kanazawa et al. 2011a/b; Wassenegger et al. 2010; COGEM 2006).

13.1 Description of the Technique
The RdDM technique is based, as its name implies, on the RNA-directed DNA-methylation process, which occurs in plants. In this process, so-called small interfering RNAs [siRNA] are formed from double-stranded RNA [dsRNA], which lead de novo to the methylation of cytosine, notably in DNA regions, which are homologous to the dsRNAs or the siRNAs (Matzke et al. 2007). If methylation occurs anew within the promoter sequences, this could lead to transcriptional gene silencing [TGS], and hence to the partial or complete inactivation of the corresponding gene (Eamens et al. 2008; Matzke et al. 2007). If methylation occurs within a silencer sequence, this can cause transcriptional activation, or, in other terms, an enhanced expression of the corresponding gene (Shibuya et al. 2009). The newly produced methylation created in these ways may be meiotically stable, and therefore passed on to offspring.

Since it is possible to trigger and to guide the plant’s own mechanism of DNA methylation with the aid of genetic engineering methods, the above-described RdDM process can be exploited for breeding epigenetically modified plants. Four different genetic engineering approaches are being currently tested and/or discussed to trigger DNA methylation with the RdDM technique: transformation; transfection; VIGS, and grafting with GM scion, or with GM rootstock (Kasai & Harada 2015; Kasai & Kanazawa 2013; Vogel 2012).

13.2 Potential Applications in Plant Breeding
With the RdDM technique, so-called epialleles can be bred in selected plants – these are alleles with an identical DNA sequence, but are expressed differently. Since the epigenetic diversity affects the phenotypic diversity and hence the plant’s performance, the use of RdDM is of interest for plant breeding. This particularly applies to those crop species with a narrow genetic base.
The technique can be used on all crop species, in which genetic engineering methods enabling the introduction of dsRNA into cells has been established (Lusser et al. 2011). Combining the RdDM technique with grafting could be of interest, especially for breeding new varieties of fruit crops (Bai et al. 2011).

13.3 Current State of Development

The concept to breed transgene-free epigenetically modified plants by means of RdDM was developed around the turn of the millennium (Wang & Waterhouse 2001). Since then, the silencing of endogenous plants by means of RdDM has been described in the following crop species: carrot (Shibukawa et al. 2009); maize (Cigan et al. 2005); petunia (Kon & Yoshikawa 2014; Kanazwa et al. 2011a/b; Sijen et al. 2001); potato (Heilersig et al. 2006); rice (Okano et al. 2008); and tomato (Kanazwa et al. 2011a). It has been demonstrated on petunia, rice and tomato that the silencing can be stably transmitted in the absence of the trigger (Kasai & Kanazawa 2013).

14. Agroinfiltration

Agroinfiltration is a technique that deploys recombinant Agrobacterium in order to achieve transient expression of genetic constructs in plant tissues. The integration of the constructs in the germ cells is not intended. The technique can be deployed in plant breeding programmes to identify plants with desirable traits. Given that cuttings and seeds of infiltrated plants can be used for further crop improvement, the question arises as to how their regulatory status is to be determined (COGEM 2006a).

If recombinant Agrobacterium that include viral vectors are deployed in Agroinfiltration, the method is called Agroinfection (Grimsley et al. 1986), or Agroinoculation (Elmer et al. 1988). Agroinfection and Agroinoculation can be assigned to the Virus-induced Gene Silencing (VIGS; Section 21), or the Virus-aided Gene Expression (VAGE; Section 22).

The floral dip method, in which flowers of a plant are dipped in an Agrobacterium suspension, can also be attributed to Agroinfiltration (Lusser et al. 2011). This particular technique, however, is not addressed here, because it leads to stably transformed plants, and hence to GMO products.

14.1 Description of the Technique

During Agroinfiltration, plant issue, mainly the leaves, is infiltrated with a liquid suspension of recombinant Agrobacterium. The infiltration can be carried out with the aid of a syringe (see Figure 5) or with toothpicks, as well as by applying a vacuum. If the roots are the target tissue, the infiltration can also succeed by merely immersing it into an Agrobacterium-suspension (Agrodrench Method, Ryu et al. 2004).

If the recombinant Agrobacterium are in the plant cells, their T-DNA will be transported into the cell nucleus, where it will lead to the transient expression of recombinant genes. The genes on the T-DNA can thereby be active as free DNA molecules; as a consequence they need not be integrated into the genome of plant cells so as to be expressed (Schaart & Visser 2009).

Depending on the genetic construct infiltrated via recombinant Agrobacterium into plant tissues, two types of Agroinfiltration can be differentiated (Lusser et al. 2011):
1. “Agroinfiltration sensu stricto”: Infiltration takes place with Agrobacterium which contains non-replicative gene constructs. The expression of the introduced genes is confined locally to the section of the plant, which has been infiltrated.

2. “Agroinoculation” or “Agroinfection”: Infiltration takes place with *Agrobacterium* containing replicative gene constructs. The genes to be inserted are first incorporated into a viral vector, which is then, in turn, integrated into the T-DNA. Since the viral vector replicates and spreads within the cells, gene expression takes place throughout the entire plant. Depending on the design of the replicative gene construct, it can lead to the formation of a protein, or to the silencing of an endogenous gene. The former case can also be attributed to VAGE (Section 22), and the latter to VIGS (Section 21).

Figure 5: Agroinfiltration of *Nicotiana Benthamiana* leaves. Source: Chandres, Wikimedia.

14.2 Potential Applications in Plant Breeding

Various applications of Agroinfiltration are possible in plant breeding\(^2\). Hence, the technology might be an interesting tool to test plants for possible disease resistance (Lusser et al. 2011; Schaar & Visser 2009). In such cases, Agroinfiltration serves as a means to select plants that could be used for further breeding. In the breeding of genetically modified plants, Agroinfiltration can be employed to test possible transgenes for a stable transformation in plant tissue (Leckie & Stewarts 2011).

Recently, Agroinfiltration (particularly Agroinoculation/Agroinfection) is also being discussed as a possible tool for the RNA-directed DNA Methylation (Section 13), for Reverse Breeding (Section 18), in Early Flowering technique (Section 15) as well as for the NRE techniques (Section 6).

\(^2\) In addition to its use in plant breeding, Agroinfiltration is also employed in basic research and molecular farming. In basic research Agroinfiltration is being used as a tool in functional genomics, or to examine the interaction between plants and pathogens (Lusser et al. 2011). In molecular farming, on the other hand, Agroinfiltration is being tested in the generation of biopharmaceutical proteins (Krenek et al. 2015).
14.3 Current State of Development

Agroinfiltration has been used in research since the 1980s. In scientific literature more than 300 relevant publications for corresponding research work are to be found (Lusser et al. 2011). In the majority of cases, the publications describe applications in basic research (Lusser et al. 2011). Regarding potential applications for plant breeding, examples can be found in the literature in which Agroinfiltration is employed for the production of recombinant proteins (Krenek et al. 2015), or for the selection of disease resistant plants (e.g. Krenek et al. 2015; Vleeshouwers et al. 2008; Zenna et al. 2006).

15. Early Flowering Technique

The Early Flowering technique is a technique to speed up crossbreeding. The genetic engineering methods deployed in the Early Flowering technique shorten a plant’s juvenile phase, whereby the breeding process can be accelerated (Schaart et al. 2015; Schaar & Visser 2009). Since the genetically engineered modification is no longer needed in the end product, plants derived from the technique can be transgene free (Schaart et al. 2015; van Nocker & Gardiner 2014; Le Roux et al. 2012; Schaar & Visser 2009).

The technique is sometimes referred to as FastTrack breeding system (Waltz 2012); high-speed breeding technique (Flachowsky et al. 2011) or accelerated breeding (Schaart & Visser 2009).

15.1 Description of the Technique

Early Flowering technique combines genetic engineering with conventional crossbreeding. The genetic engineering methods are thereby used to induce early flowering in a selected plant. The early flowering plant is then crossbred with another plant.

Traits that can be introgressed by the use of the Early Flowering technique could originate, at least theoretically, in the primary, secondary or tertiary gene pool. Hence, not only can pre-existing varieties, landraces or sexually compatible wild species act as a source of genes, but also non-sexually compatible species can do so. The latter is possible in two ways: First, by introgressing the desired genes from a non-crossable species via bridge crossing in a sexually compatible species prior to initiating the Early Flowering technique. Second, by transferring genes from the tertiary gene pool via embryo rescue into the primary gene pool prior to the start of the Early Flowering technique.

Inducing early flowering can be achieved either through the expression of “flower induction genes” (e.g. BpMADS4), or by the silencing of endogenous genes (e.g. TFL1) by means of RNAi technique (Schaart & Visser 2009). Four different genetic engineering approaches are currently being tested or discussed to achieve the expression, or the silencing of these genes (Schaart et al. 2015; van Nocker & Gardiner 2014; Vogel 2012): Transformation, VIGS (Section 21); VAGE (Section 22); and Grafting on GM Rootstock (Section 4.2). Figure 6 presents a simplified schema of the Early Flowering technique for the case in which early flowering is triggered by transformation.
Figure 6: Simplified breeding schema where early flowering is induced by means of stable transformation to introgress a desirable genetic trait from a wild species in an elite variety (as per Flachowsky et al. 2009). The breeding schema consists of the following steps: (1) The plant variety, into which the desirable genetic traits are to be introgressed are genetically engineered to flower earlier than they would naturally do. (2) The GM plant is crossed with the plant containing the desirable genetic trait. (3) The crossbred offspring (filial generation 1, or, in short, F1) are selected, where with those individuals chosen for further breeding will contain the inserted genes for early flowering as well as the desirable genetic traits. (4) The selected F1 plants are crossbred with the genetically non-modified parental plant, in which the desirable genetic traits should are to be introgressed. (5) The offspring of the pseudo-backcross generation (BC1) are selected, whereby, in turn, those selected for further breeding contain both the inserted genes for early flowering as well as the desirable genetic trait. (6) The selected BC1 plants are, in turn, crossbred with the genetically non-modified parent plant, in which the desirable genetic traits are to be introgressed. (7) The pseudo-backcross is repeated so often until the undesirable genetic traits (linkage drag), which have been introduced along with the desirable traits, are gradually removed. (8) Once the linkage drag is sufficiently removed by backcrossing, plants having the desirable genetic traits, and not the genetically engineered constructs, are selected in the final stage.

15.2 Potential Applications in Plant Breeding
The Early Flowering technique is of particular interest for the breeding new varieties of fruit and forest trees. Compared with breeding varieties in annual plants, producing new tree varieties takes a considerably longer time. Due to the protracted juvenile phase for fruit and forest trees, certain traits can only be realised in new
varieties with the use of classical crossbreeding methods after many decades, or, perhaps even not at all (Flachowsky et al. 2011; Fladung 2011). For example, to breed a new variety of apple that would exhibit the genetic traits of wild apples, more than 50 years of breeding work may be necessary (Flachowsky et al. 2011). Using the Early Flowering technique enables a reduction in the trees’ juvenile phase to several months, thereby precipitating its first flowering; hence time can be saved in the breeding process.

In addition to its use in breeding new fruit- and forest tree varieties, the Early Flowering technique is also used in annual plants in order to shorten the breeding process (Yamagishi & Yoshikawa 2011a/b).

15.3 Current State of Development

In 1995, it was first demonstrated on aspen that the juvenile phase of trees could be shortened through the use of genetic engineering methods. Since then the technique has been steadily refined, further developed and tested on other tree species. Among the trees species on which research projects have deployed the Early Flowering technique are: apple (Weigl et al. 2015; Kishigami et al. 2014; Yamagishi et al 2014/2011; Wenzel et al. 2013; Le Roux et al. 2012; Flachowsky et al. 2011/2007/2006; Yamagishi et al. 2011; Tränkner et al. 2010; Zhu et al. 2009; Kotada et al. 2006); aspen (Weigel & Nilsson 1995), bitter orange (Endo et al. 2005); citrus trees (Cervera et al. 2009; Pena et al. 2001); eucalyptus (Klocko et al. 2016); kumquat (Duan et al. 2010); olive (Cerezo et al. 2014); pear (Freiman et al. 2012; Matsuda et al. 2006); plum (Srinivasan et al. 2012); poplar (Hoenicka et al. 2014; Tränkner et al. 2010; Zhang et al. 2010; Böhlenius et al. 2006; Hsu et al. 2006); and rugosa rose (Xing et al. 2014b).

Field tests with early flowering trees have been carried out on apple and plum in the USA (ISB 2015; Scorza et al. 2014) as well as on citrange and orange in Spain (JRC 2015).

Research projects for annual plants have been carried out on cotton (McGarry & Krager 2013; McGarry & Ayre 2012); soybean (Yamagishi & Yoshikawa 2011 a/b); and tobacco (Lewis & Kernodle 2009).

16. Marker Gene-aided Haploid Breeding

For the purpose of this report, Marker Gene-aided Haploid Breeding refers to a technique that facilitates the generation of haploid plants, by transforming existing haploid inducer lines with a visually selectable marker gene. Since the marker gene is no longer present in the resulting haploids, plants derived from the application of the technique can be transgene free.

16.1 Description of the Technique

The technique combines in-vivo haploid induction with genetic engineering methods. The in vivo haploid induction is based upon the use of so-called inducer lines; these are plants, which – used as pollinators – give rise to haploid progeny. A crucial and laborious step in the application of the technique consists of differentiating between haploid and diploid offspring. In order to simplify this step, inducer lines can be transformed with a gene that codes for the green fluorescent protein [GFP]. Differentiating the offspring will be facilitated with the use of such GFP inducer lines because only diploid offspring produce GFP. Since haploid offspring do not harbour the gene for GFP, plants derived from the application of the technique can be transgene free (Yu & Birchler 2015; Palumbo 2003).
16.2 Potential Applications in Plant Breeding
The technique can be used for the generation of haploids in those crop species in which haploid inducer lines are available.

16.3 Current State of Development
Reports on the application of the technique are to be found for *maize* (Yu & Birchler 2015); *potato* (Palumbo 2003); and *tobacco* (Hancock et al. 2015).

17. Marker Gene-assisted Crossbreeding
For the purpose of this report, Marker Gene-assisted Crossbreeding refers to a technique that facilitates the crossing of plants from different species or genera by equipping one of the crossing partners with a selectable marker gene (Heffelfinger et al. 2015; Kausch et al. 2013). Since the marker gene is required only during the breeding process, the resulting plants can be free from recombinant DNA (Heffelfinger et al. 2015; Kausch et al. 2013). A variant of the technique is referred to as the in-situ embryo rescue (Hague et al. 2014; Tilelli et al. 2012).

17.1 Description of the Technique
The crossing of individual plants belonging to different species or genera is a long-established practice used to obtain new breeding material. This procedure, known as Wide Crosses, is extremely laborious and time-consuming, because it is difficult to select hybrid progeny, and/or the embryo rescue technique must be applied. The Marker Gene-assisted Crossbreeding offers an option to facilitate Wide Crosses. It is based upon the fact that one of the plants to be crossed is genetically equipped with a selectable marker – for example, with a gene for herbicide tolerance. Hence, this makes it possible to select hybrid offspring and/or to facilitate embryo rescue, because immature embryos can be placed directly in culture media without removing their nutritive tissue (Kausch et al. 2013). Hybrid offspring that are fertile can then be backcrossed with untransformed parental plants, whereby the transgenic marker gene can be segregated (Kausch et al. 2013).

17.2 Potential Applications in Plant Breeding
Marker Gene-assisted Crossbreeding simplifies Wide Crosses and thereby facilitates the introgression of genes into a crop species from those species, which are difficult to cross. The technique is interesting in those cases in plant breeding, in which the genetic variation within a particular crop is insufficient to achieve improvements in breeding.

17.3 Current State of Development
Marker Gene-assisted Crossbreeding is currently being applied on switchgrass (Heffelfinger et al. 2015; Hague et al. 2014; Kausch et al. 2013; Tilelli et al. 2012). As of the time this report-update is being prepared, it is not known whether the technique is also being tested on other crops.
18. Reverse Breeding [RB]

Reverse Breeding [RB] is a novel plant breeding technique which uses genetic engineering methods to suppress meiotic recombination, and in so doing facilitate the breeding of F_1-hybrids (Dirks et al. 2009/2003). Since the genetically engineered modification is no longer needed in the final product, transgene free plants can be obtained with Reverse Breeding (Lusser & Davies 2013; Lusser et al. 2012/2011; Chan 2010; Dirks et al. 2009; Wijnker & de Jong 2008).

18.1 Description of the Technique

The RB technique consists of a combination of genetic engineering methods, techniques for producing doubled haploid plants (DH technique), tissue culture techniques and conventional crossbreeding methods. Genetic engineering is used to suppress meiotic recombination in a selected heterozygous plant. This can be achieved by silencing the expression of plant genes involved in the meiotic recombination process (Schaart & Visser 2009; COGEM 2006a). To silence these genes, three genetic engineering approaches are under consideration (Vogel 2012; Dirks et al. 2009): Transformation; VIGS (Section 21); and Grafting on GM Rootstocks (Section 4.2). Figure 7 illustrates the most important steps for the case in which the meiotic recombination is suppressed by means of transformation.

Figure 7: Schematic representation of Reverse Breeding technique according to Wijnker & de Jong (2008). The starting plant is a fictive F_1 hybrid with three chromosomes pairs (2n=2x=6). Meiotic recombination is suppressed by transforming the F_1 with an RNAi construct. The RNAi construct leads to silencing one of the genes required for the formation of crossovers. The red dots represent the inserted RNAi constructs. The achiasmatic meiosis leads to spores, which carry non-recombinant chromosomes. Most of the resulting spores are unbalanced (not shown in the diagram); some spores, however, are balanced (several options drawn). Doubled-haploid plants are produced from balanced spores. Among the doubled haploids plants, reciprocal genotypes [Parent 1 and Parent 2], which no longer carry the RNAi construct and which yield the original F_1 hybrid after crossing can be selected. Parent 1 and Parent 2 are derived from two different primary transformants, which carry the RNAi-construct on different chromosomes. Source image by: Wijnker & de Jong (2008).
18.2 Potential Applications in Plant Breeding

Two potential applications in particular of Reverse Breeding are discussed in the scientific literature: production of F₁-hybrids for cultivation, and production of chromosomal substitution lines for further breeding (Lusser et al. 2011; Chan 2010; Dirks et al. 2009; Wijnker & de Jong; 2008 Forster et al. 2007). These applications are possible in plant species having no more than 12 chromosomes, and in which the DH technique can be applied (Lusser et al. 2011 Dirks et al. 2009).

The most important function of the RB technique is to conserve elite genotypes, or to produce F₁-hybrids. Since with Reverse Breeding homozygous parental lines can be generated for any desirable heterozygous plant, it is also possible to preserve uncharacterised heterozygote genotypes with elite properties. This is unlikely to be achieved with traditional plant breeding methods (Dirks et al. 2009; Wijnker & de Jong 2008). Reverse Breeding facilitates and accelerates the breeding of F₁ hybrids (Schaart & Visser 2009).

In addition to the production of F₁-hybrids, Reverse Breeding can also be used to generate chromosome substitution lines. These lines contain one or more chromosomes of one parent in the genetic background of the other parent. Backcrossing, for example, could result in hybrids that are heterozygous for all but one chromosome, or are homozygous for all but one chromosome (Dirks et al. 2009). Chromosomal substitution lines can for be used for genetic studies as well as for improving parental lines (Lusser et al. 2011; Chan 2010 Wijnker & de Jong 2008).

18.3 Current State of Development

The RB technique concept was initially described in 2003 (Dirks et al. 2003) and its feasibility was demonstrated on the model plant Arabidopsis in 2012 (Wijnker et al. 2012). While scientific literature on the topic does discuss the approach and potential applications of the RB technique (Barabaschi et al. 2016, Crismani et al. 2013, McGarry & Kragler 2013; Chan 2010; Dirks et al. 2009; Wijnker & de Jong 2008; Forster et al. 2007), current examples of concrete application on crop plants are as yet to be found.

19. Seed Production Technology

Seed Production Technology [SPT] is a process in which GM maintainer lines are employed to propagate male-sterile parental lines, which can be used in the production of hybrid seed (Wu et al. 2016; Whitford et al. 2013; FSANZ 2013; Shenoy & Sharma 2012). The final product, i.e. the hybrid seed, no longer contains the maintainer line’s genetic modifications (Wu et al. 2016). Since application of SPT doesn’t require female parental lines being detasseled, higher yields and better quality can be achieved in the production of hybrid seed.

19.1 Description of the Process

SPT is based on the use of two different inbred lines: a male-sterile female line and a GM maintainer line, having the same genetic background as the mother line. The male-sterile female line functions as the female parent in the production of hybrid seed. Since it is male-sterile, it cannot fertilise itself; this is why detasseling, which is normally necessary in hybrid seed production, is omitted. In order to propagate seed of the male-sterile parental line, the GM maintainer line is used. This line carries a construct containing three transgenes: (I) a gene for a male fertility protein expressed in the anthers; (II) an alpha-amylase gene active in the pollen grains; as well as (III) a dsRed gene active in the seeds. Unlike the male-sterile mother line, the maintainer line can
produce pollen. Half of pollen produced contains the introduced transgene construct, and is infertile. The other half of the pollen does not contain the transgene construct and is fertile.

The SPT process consists of three essential steps: 1. propagating the maintainer line; 2. propagating the male-sterile mother line; 3. producing hybrid seed destined for commercial use.

The maintainer line can be readily propagated by self-pollination. The resulting seeds are either red or yellow, but only the red seeds contain the inserted gene construct and are selected for propagating the male-sterile female line. Separating the red and yellow seeds is carried out mechanically. During propagation of the mother line, the non-transgenic pollen of GM maintainer-line ensures that pollination occurs. The resulting seeds can, in turn, be sorted by machine into yellow and red seeds. The yellow, non-transgenic seeds will eventually be sown with the seed of a male-fertile elite inbred line in a hybrid seed production field. The field’s harvest consists of seeds destined for sale as hybrid seeds.

The descriptive details of the SPT process are taken from the USDA (2011) and Waltz (2012).

19.2 Potential Applications in Plant Breeding

SPT is not actually a breeding technique but rather a process that renders hybrid seed production more efficient. Efficiency will be increased because detasseling the mother line – often necessary in hybrid seed production – ceases to be a necessary step. Even though the SPT process was originally developed for the production of hybrid maize seeds it can equally be applied to other crop species (Wu et al. 2016; FSANZ 2013).

19.3 Current State of Development

Commercial hybrid seed production using the SPT process is currently feasible for maize in the USA. In 2011, the United States Department of Agriculture [USDA] announced its determination of non-regulated status for the SPT maintainer line DP-32138-1 of DuPont Pioneer (USDA 2011), which allows planting of the transgenic line for hybrid seed production. Since 2012, DuPont Pioneer has been deploying DP-32138-1 in US maize hybrid seed production operations (Wu et al. 2016). The total acreage grown with DP-32138-1 is presumed to be less than 2,000 hectares per year. DuPont Pioneer is currently broadening the scope of the SPT process to rice (field tests in India) and wheat (proof of concept experiments).

20. Centromere-mediated Genome Elimination

The Centromere-mediated Genome Elimination technique deploys genetic engineering methods to produce haploid inducer lines, hence enabling the production of doubled haploid plants (Tek et al. 2014; Ravi et al. 2014; Ravi & Chan 2013/2010). Since the genetically engineered modifications of the inducer line is no longer needed in the final product, transgene free plants can be obtained (Murovec & Bohanec 2011; Chan 2010).

The technique is sometimes referred to as the CCE technique [centromere-mediated chromosome elimination technique].( Camacho et al. 2014).

20.1 Description of the Technique

Genetically engineered haploid inducer lines are produced during the application of the Centromere-mediated Genome Elimination technique (Chan 2010). If non-GM plants are crossed with these GM inducer-lines, this will result, inter alia, in progeny that are haploid and only contain chromosomes of the non-GM plants. Doubled
haploid plants can either spontaneously result from the haploid plants, or following the application of chromosome-doubling methods.

20.2 Potential Applications in Plant Breeding
The Centromere-mediated Genome Elimination technique offers a novel option to produce doubled haploid plants (Chan 2010; Copenhaver & Preuss 2010). Doubled haploid plants play an essential role in breeding, because traits can be fixed without the need to backcross over several generations (Dwivedi et al. 2015; Ferrie & Möller 2011).

Since the technique enables the transfer of parental chromosomes into a maternal cytoplasm, plants with cytoplasmic male sterility can also be generated (Chan 2010). In addition, the ploidy in polyploided plants could be halved through use of the Centromere-mediated Genome Elimination technique. A potential combination with the Reverse Breeding technique has also come up for discussion (Stower 2012; Wijnker et al. 2012).

20.3 Current State of Development
The feasibility of the Centromere-mediated Genome Elimination technique was first demonstrated on the model plant *Arabidopsis* in 2010 (Ravi & Chan 2010). Since then, no publications on applications of the technique have appeared in the scientific literature. Nonetheless, the technique has aroused great interest in the plant breeding community, (Dwivedi et al. 2015; Tek et al. 2015; Bhat 2011; Brownfield & Köhler; 2011 Segui-Simarro et al. 2011; Marimuthu et al. 2011; Chan 2010; Copenhaver & Preuss 2010) and is currently being tested on several crop species, including banana, barley, manioc, soybean, sugar beet and switchgrass (Tek et al. 2015).

21. Virus-induced Gene Silencing [VIGS]

Virus-induced Gene Silencing [VIGS] is a technique that employs plant viral vectors so as to silence the expression of endogenous plant genes (Schaart & Visser 2009). The technique is used primarily as a tool for functional genomics and reverse genetics (Di Stilio 2011; Purkayastha & Dasgupta 2009), but is also employed in conventional as well as marker-assisted breeding (Senthil Kumar & Mysore 2011b). Recently, VIGS is also being discussed as a tool for NPBT (Senthil-Kumar & Mysore 2011b), specifically in connection with RNA-directed DNA-Methylation (Kanazawa et al. 2011a/b; Section 13); with Reverse Breeding (Dirks et al. 2009; Section 18); and with Early Flowering technique (Sasaki et al. 2011; Section 15).

21.1 Description of the Technique
VIGS uses recombinant viral vectors in order to silence endogenous plant genes. The viral vectors are not thereby introduced into germ cells, but rather into somatic cells. A stable transformation of the plant is not intended.

VIGS is fundamentally based on the plant’s own process of siRNA mediated RNA silencing. In this process, so-called small-interfering RNAs [siRNA] are formed from double-stranded RNA [dsRNA], leading to specific degradation of messenger RNAs [mRNAs] with homology to dsRNA/siRNA. Degradation of the mRNA leads to post-transcriptional gene silencing [PTGS] of the corresponding genes. In addition to initiating PTGS, VIGS can also be used to trigger the plant’s own process of RNA-mediated DNA methylation, and hence to induce transcriptional gene silencing [TGS] (Kanazawa et al. 2011a/b; Otagaki et al. 2011; Jones et al. 1998). In these cases, the siRNAs result in cytosine residues being methlyated, specifically in genomic regions, which are
homologous to the dsRNAs/siRNAs. If methylation happens \textit{de novo} within promotor sequences, this may lead to the TGS of the corresponding gene (Matzke et al. 2007; Eamens et al. 2008).

In order to exploit the processes described above for the VIGS-technique, plants are inoculated with recombinant viral vectors harbouring fragments from the host plant gene to be silenced.

VIGS consists essentially of three stages: (I) preparation of recombinant vectors; (II) introducing vectors into the plant, and (III) silencing of the target gene. If the treated plant material is used for further breeding, a fourth stage will follow, which consists in removing the VIGS vector.

Both RNA- as well as DNA-plant-viruses can be used to produce recombinant vectors (Senthil-Kumar & Mysore 2011b; Purkayastha & Dasgupta 2009). It is also possible to use the satellite virus-induced silencing system, SVISS; (Gossele et al. 2002). Of particular interest are viruses or vectors that can be used in several plant varieties, and which are weak or attenuated in such a manner that they provoke few or no symptoms of disease in plants.

Introducing the vectors into the plant can be achieved using various methods, whereby the choice of method is contingent upon the plant variety and the vector. Possible methods include: biolistic techniques; mechanical inoculation of \textit{in-vitro} transcribed RNA; inoculation of RNA from leaf extracts of infected plants as well as Agroinfection and Agroinoculation (Stratmann & Hind 2011; Purkayastha & Dasgupta; 2009 Robertson 2004).

In the latter two methods, the viral vectors are introduced into T-DNA vectors, which, in turn, are used for the transformation of \textit{A. tumefaciens} (see Section 14).

In the vast majority of cases, VIGS is temporary and usually lasts between 2 and 16 weeks. However, it can also persist throughout the plant’s entire lifespan, and can be – if the viral vector is transferred, or as in the case of TGS – transmitted to the offspring (Senthil-Kumar & Mysore-2011a/b; Becker & Lange 2010).

Various methods can be used to select virus-free offspring, whereby the choice of method depends on how the chosen plant propagates itself, and whether the virus used can be transmitted through seeds or not (Senthil-Kumar & Mysore 2011b; Schaat & Visser 2009). In the case of vegetatively propagated plants, viruses can be successfully eliminated with specific techniques such as thermotherapy; cryotherapy and meristem cultivation (Senthil-Kumar & Mysore 2011b; Schaat & Visser 2009; Wang & Valkonen 2009/2008; Wang et al. 2008). In sexually propagated plants, the seeds can still be used if the virus introduced is not, or not completely, transmitted through seeds (Senthil-Kumar & Mysore 2011b; Schaat & Visser 2009).

\section*{21.2 Potential Applications in Plant Breeding}

VIGS is mainly used in basic research, specifically as a tool for functional genomics and reverse genetics (Becker & Lange 2010; Purkayastha & Dasgupta 2009; Robertson 2004). In plant breeding, VIGS can be used to screen for plants with desirable traits. In addition, VIGS is discussed as a breeding tool for RNA-directed DNA methylation (Kanazawa et al. 2011a/b; see section 13); in Reverse Breeding (Dirks et al. 2009; see Section 18); and in Early Flowering technique (Sasaki et al. 2011; Senthil-Kumar & Mysore 2011b; see Section 3.6).

\section*{21.3 Current State of Development}

The use of VIGS started in the mid-1990s (Kumagai et al. 1995). Since then the technique has been continually elaborated upon and further developed. Currently, more than 30 different VIGS vectors are known, which, in all, have been applied to a total of more than 40 plant species (Senthil-Kumar & Mysore 2011b).

If, and to what extent, VIGS is currently employed in plant breeding programmes, is not apparent from the documents reviewed.
The deployment of VIGS as a tool for NPBT is currently being tested in the Early Flowering technique and for RNA-directed DNA Methylation (Kanazawa et al. 2011a/b; Sasaki et al. 2011; Senthil-Kumar & Mysore 2011b).

22. Virus-aided Gene Expression [VAGE]

Virus-aided Gene Expression [VAGE] is a technique in which plant viral vectors are employed to temporarily express newly introduced genes in plants. The technique is largely identical with VIGS (see Section 21) with the notable difference that with VAGE the viral vectors do not contain RNAi-constructs but rather expression cassettes. VAGE has been partially tested as a tool for NPBT, particularly in the NRE technique (Ali et al. 2015a/b; Mahfouz & Li 2011; Vainstein et al. 2011; Marton et al. 2010; Section 6) and in the Early Flowering technique (Yamagishi et al. 2011; Yamagishi & Yoshikawa 2011a/b; Section 15).

23. Transformation with wild-type Agrobacterium rhizogenes

Transformation using wild-type Agrobacterium rhizogenes is a breeding technique, also referred to as “natural transformation” in the scientific literature, with which the T-DNA of the Ri-plasmid of the bacteria is transferred in an unmodified form into the plant genome (Kuligowska et al. 2015; Lütken et al. 2015). Although the technique does not require the use of recombinant DNA, the resulting plants harbour foreign genes and hence can be deemed as transgenic. Accordingly, clarification may be needed as to whether the resulting plants are GMOs from a legal perspective.

In Denmark, new compact varieties of Kalanchoe are being developed through the application of the technique (AgroTech 2015). In Italy, field releases of cherry rootstocks transformed with wild-type Agrobacterium rhizogenes have taken place (Rugini et al. 2015).


24.1 Epigenetic Editing

Epigenetic Editing is currently being discussed by several authors as a potential NPBT that could be deployed for a targeted modification of the epigenome of plants (Bortesi & Fischer 2015; Kumar & Jain 2015; Fichtner et al. 2014; Zhang & Hsieh 2013). The concept of Epigenetic Editing is similar to that of the NRE technique (Section 6). Unlike with the NRE technique, site-specific nucleases are not used, but rather enzymes that are produced by means of a fusion of programmable DNA-binding domains [ZF, TALE or dCas] with chromatin-modification domains (e.g. methylases, demethylases or histones). The concept is based upon the idea that such synthetic enzymes should be capable of producing epigenetic alterations at predetermined genomic sites in plants. The functionality of such enzymes has already been demonstrated outside the plant kingdom (Keung et al. 2015; Köeferle et al. 2015).
Given that methylation patterns newly-generated with the synthetic enzymes can be stably inherited, the presence of those genes that encode the synthetic enzymes in the plant would only be temporarily necessary for the production of epialleles. Hence, Epigenetic Editing could produce transgene free plants.

24.2 Transgene-based Recurrent Selection
Transgene-based Recurrent Selection is a technique proposed by Tanaka (2010), which could enable breeders to enrich specific genes in a population of autogamous crop plants without restricting the genetic basis. The concept is based on genetically engineering plants in such a way that they exhibit both a positively and negatively selectable male sterility. Since the selectable transgenes are no longer needed in the final product, the plants derived from the application of this breeding technique can be transgene free (Tanaka 2010).

24.3 Targeted Chemical Mutagenesis
Targeted Chemical Mutagenesis has been discussed as a potential NPBT by COGEM (2006). The concept underpinning the technique is to couple mutagenic substances onto oligonucleotides in order to generate mutations at predetermined genomic sites. The function of the oligonucleotides consists of directing the mutagens to the target sequence in the genome. To date, no concrete examples of the application of the technique on plants have been found in the literature.

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3 The web-sites for each and every reference noted herein were last visited on 20 December 2015.


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