

# New Breeding Techniques and Their Possible Regulation

**Esra Seyran and Wendy Craig**

*Biosafety Group, International Centre for Genetic Engineering and Biotechnology (ICGEB), Italy*

New breeding techniques (NBTs) are gaining greater uptake in plant breeding programs around the world, due to their greater precision and potential to reduce varietal development times. As the first products of research begin to enter the commercial domain, some of their technical and conceptual overlaps with GM biotechnology have become the focus of international discussions concerning their regulatory status. This review provides an insight into the mechanisms of NBTs, how their products may/may not differ from existing plant products which themselves may/may not be subject to government regulation, and whether a case can be made for them to fall under/escape GMO regulatory oversight. What is especially obvious is that until there is certainty of their regulatory status in key territories and regions, innovation in plant breeding risks stagnation, and both costly delays in market rollouts and trade disruptions are likely due to incompatible and non-harmonious regulatory practices and policies.

**Key words:** biosafety, biotechnology, genetically modified organisms, government regulation, mutagenesis, new breeding techniques, plant breeding.

---

## Introduction

By 2050, the Food and Agriculture Organization of the United Nations (FAO) estimates that the global population will reach 10 billion people and predicts that global food production will have to increase by 70% in order to cope with the resulting food security challenges (FAO, 2009). At the same time, increasing environmental pressures on arable land, the quality and availability of water, and the decline in biodiversity continue to challenge sustainable food production (FAO, 2015). The world is in need of new strategies to meet these new challenges. The emergence of novel strains of pathogens has created significant challenges to crop production, and farmers have had to counter the resulting heavy disease pressures with the use of pesticides (Cools & Hammond-Kosack, 2013). New approaches to developing durable resistance in crops are urgently needed. It is not certain how climate change will affect global agricultural productivity, but it is generally accepted that improved crop traits will also be required to adapt to variable local conditions (Fears, Aro, Pais, & ter Meulen, 2014). According to the Organization for Economic Cooperation and Development (OECD) and the FAO, the main food crops are still below their potential yield in many regions, with yield gaps in excess of 50% in many developing countries (FAO, 2009; OECD, 2016). Through advances in plant breeding, annual yield gains of 1-2% have been achieved in a number of crops, allowing global food production to increase by 25%

between 1990 and 2000 (European Academies Science Advisory Council [EASAC], 2004, 2011). The world continues, however, to face significant challenges to increasing agricultural nutritional quality and output, and overcoming these complex challenges will require versatile solutions. Genetic crop improvement can contribute towards addressing these problems.

In 2016, the global area cultivated with genetically modified (GM) crops reached 181 million hectares. The leading countries, in terms of total cultivated area of GM crops, were the United States, Brazil, Argentina, Canada, and India, respectively. The contribution of developing countries to the total area of GM crop cultivation is increasing every year and reached 54% of global GM crop cultivation in 2016. The most cultivated GM traits are currently herbicide tolerance and insect resistance (James, 2016). Around the world, legislation for genetically modified organisms (GMOs) is based on the principle of assuring the safety and health of humans, animals, and the environment. However, practical implementation differs from country to country. For example, in Argentina, the EU, Japan, and South Africa the definition of a GMO is based on the techniques used to generate it, whereas Canada and United States have not established legal frameworks specifically for the regulation of GMOs. Instead, Canada uses its regulation of plants with novel traits, while the United States regulates GMOs under the laws and guidelines that cover food, animal feed, drugs, pesti-

cides, plant pests, and toxic substances (Lusser, Parisi, Plan, & Rodríguez Cerezo, 2012).

Food security is a multifaceted problem that needs multifaceted solutions. Plant breeding is one of the best means of achieving food security owing to its potential to improve pre-harvest, harvest, and post-harvest performances. All methods for plant genetic improvement should be available for consideration and the most appropriate selected based on considerations of trait(s), the crop, the environment, and the socio-economic setting in which the crop is to be grown. Governance and regulatory systems are essential to assess the risks and benefits of each plant genetic improvement technology, and to make these assessments available to the wider regulatory and regulated community, as well as for public education and reassurance. It should be kept in mind that breeding techniques are generally complementary and not mutually exclusive, that all are essential tools in addressing the challenges of agriculture, and that, as in all of life's activities, no action, including plant breeding and its techniques, is risk-free (Schaart & Visser, 2009).

Genetic changes resulting from conventional breeding range from small changes in the DNA sequences to combining entire genomes through hybridization. Although conventional breeding is well-established, it is slow and limited to the genetic diversity of current varieties or their compatible relatives, moreover the co-introduction of unwanted genetic traits is inevitable. Mutation breeding techniques, such as ionizing radiation or chemical mutagenesis, have been used for decades in conventional plant breeding programs to generate desirable traits for crop improvement. Over the years, such means of inducing mutations have yielded productive results, but again require time-consuming and expensive screening of large populations, taking several generations to reach the final result. Genetic modification via transgenesis allows the introduction of a wider range of traits not available through conventional or mutational breeding, and it is most useful for traits that depend on a small number of known genes; however, many desirable traits are the result of complex interactions of several gene products (Jander et al., 2003; Till et al., 2007).

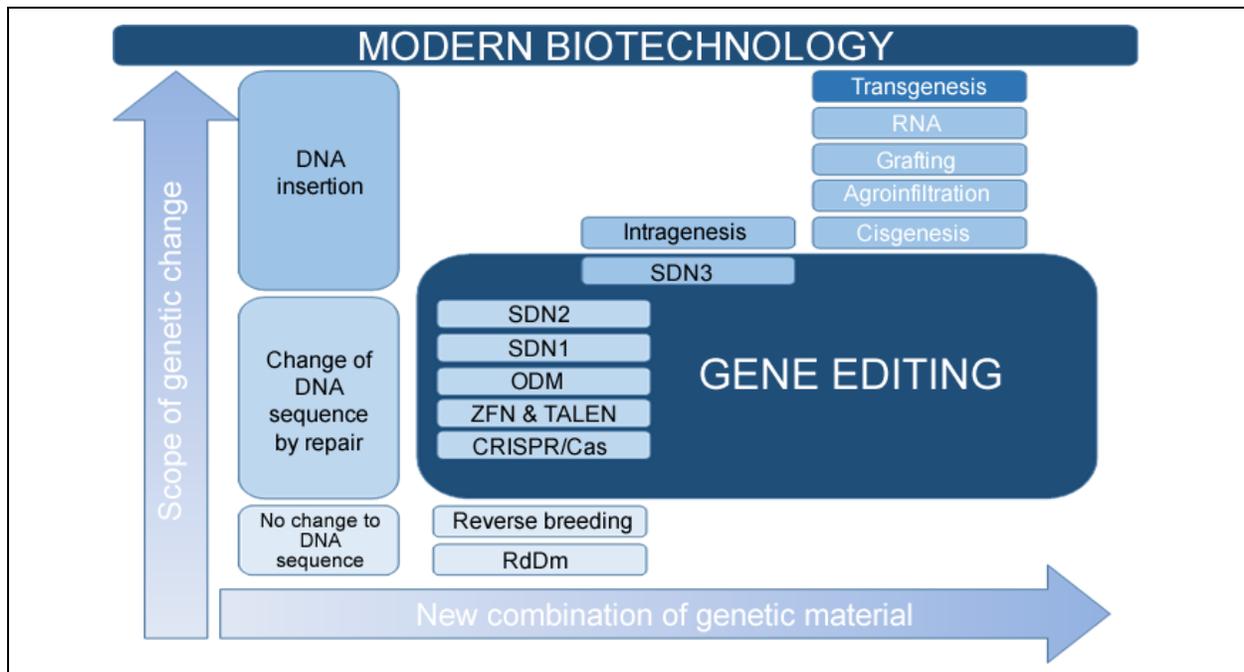
### **The Continuing Evolution of Plant Breeding**

Our continuously advancing understanding of plant biology and genetic diversity provides new opportunities for the development of plant varieties that are better adapted to meet the challenges that agriculture faces

today and in the future. Next-generation DNA sequencing (Edwards, Batley, & Snowdon, 2012) and developments in high-throughput genome assembly and analysis are advancing our understanding of the intricacies of plant genomes (Jorriñ-Novó et al., 2009), while proteomics (Morrell, Buckler, & Ross-Ibarra, 2012), transcriptomics (Jiao et al., 2009), and metabolomics (Fernie, 2009) improve our insights into cell function and development.

The so-called “new breeding techniques” (NBTs) offer the possibility of making genetic changes more precisely than was previously possible by targeting them to specific sites in the genome. These new tools are essential to maximize the potential of crops, and to address the challenge of limited genetic diversity for important traits such as resistance to pests, efficient use of water and nitrogen, drought tolerance, and improved nutritional content and taste (Lusser et al., 2011). Conventionally, breeding techniques rely on the sexual crossing of parental lines to obtain the desired phenotypes. Plant breeders choose progenies with the desired trait and backcross them to one of the parental lines for several generations to remove any co-crossed undesired traits. This process usually takes several years, depending on the crop, before the expression of the desired trait can be fixed and further expanded into commercial varieties. In addition to this long generation time, conventional breeding relies heavily on the genetic variation and sexual compatibility available within the gene pool. Further, many plants are polyploid, carrying multiple copies of the genome per cell (e.g., six copies in wheat, seven in bananas), each of which must be changed during the breeding process, thus further slowing the process of fixing desired traits. Certain NBTs such as CRISPR/Cas (see Box 1) can simultaneously improve all copies of the target gene in a controlled fashion (Wang et al., 2014).

NBTs have the potential to reduce the cost and time of bringing new products to the market since, compared with conventional breeding techniques, they can reduce the number of unwanted traits that might be co-transferred during the breeding process and that subsequently need to be removed (Schaart & Visser, 2009). The greatest potential advantages of NBTs are their relative ease, precision, speed, and low cost, allowing breeders to focus more on the local growing conditions and to react more quickly to the changing needs and wants of growers and consumers. Conventional breeding techniques, and some of the initial methods of producing GM crops, require the maintenance and testing of large numbers of plants over periods up to several years, depending on the



**Figure 1. A compilation of NBTs mentioned to date in different regulatory analysis, ranked according to the scope of genetic change.**

*Modified from Lema (2017), with permission.*

crop, before the best candidate can be selected for varietal registration. Because NBTs use very specific and defined mechanisms to introduce wanted traits with much higher selectivity, the number of test subjects can be significantly reduced. Furthermore, several different genes can be modified concurrently for every gene copy in a given genome, even in polyploid species, as mentioned above (Jiang et al., 2013). Thus, consumer benefits such as enhanced nutrient content, prolonged shelf life, or improved color, odor, flavor, and texture of the plant can be incorporated simultaneously with producer benefits, such as improved pest resistance or yield (Bortesi & Fischer, 2015).

The development of breeding techniques has continued to progress rapidly, resulting in even more sophisticated methods to create plants with new traits. After three decades of genetic engineering in plant breeding, most applications have yet to result in complex genetic changes. NBTs (see Box 1) are able to manipulate the genome and gene regulation in a targeted way, while minimizing undesirable side effects.

### The Regulation of Biotechnology-derived Plants

By the 1990s, applications of biotechnology methods in plant breeding resulted in new regulations and governance for the development and release of GMOs in specific countries. However, continuous advancements in the area of plant biotechnology—and particularly the developments over the last 10 years—have led to the widespread availability of NBTs that are not yet clearly defined in current GMO regulatory frameworks. Therefore, uncertainty concerning the regulatory status of the products of NBTs remains a major obstacle to their commercialization. Specifically, for regulatory purposes, will the products of NBTs be classified under the current definitions of genetic modification? In a broad sense, NBTs can be categorized as those (Figure 1)

- that employ targeted mutagenesis,
- in which transgenes are used in the early stages of breeding but the final plants are free of transgenes, or
- that employ DNA from foreign or cross-compatible species and utilize one of the standard DNA transformation techniques.

The “amount” of resultant genetic change could be a paramount criteria to define the regulatory status of a particular NBT, depending on the specific definition of GMO used; for instance, in the Cartagena Protocol on Biosafety’s (CPB) definition of living modified organisms (LMOs),<sup>1</sup> the key concept is “novel combination of genetic material” (Lema, 2017). Obviously, NBTs offer both advantages and challenges, and the pertaining regulatory framework will certainly have an impact over whether the potential advantages of such NBT-derived products can be realized.

The advancements in recombinant DNA technology since the 1980s have facilitated the development of transgenic plants as a complement to, or an extension of, conventional plant breeding. The major international instrument on LMOs—the CPB—was adopted in 2000 and became effective in 2003 (Secretariat of the Convention on Biological Diversity, 2000). The Protocol mandates “the safe handling, transport and use of living modified organisms (LMOs) resulting from modern biotechnology, that may have adverse effects on biological diversity, taking also into account risks to human health.” Decisions authorizing the transboundary movement of LMOs under the CPB are to be based on a risk assessment, as outlined in Annex 3 of the Protocol, which provides the minimum requirements needed to be fulfilled by the parties in this respect. This requires that these risk assessments

- be case-specific (case-by-case approach),
- identify differences between the LMO in question and its comparator (comparative approach),
- identify any necessary risk-management measures and strategies, and
- be redone should any new relevant information become available at a later date (iterative approach).

Recent advances in the field of agricultural biotechnology have left most governments unsure whether the products of NBTs should be regulated (Jones, 2015), with much of the debate being polarized. Those stakeholders who share the view that the new techniques should be exempt from GMO legislation generally argue that the end-products are very similar, if not identical, to

products generated using conventional techniques. Those with the opposing view contend that the new techniques are very similar to those used to create GMOs and thus should be regulated similarly.

The OECD working group on harmonization of regulatory oversight in biotechnology has published a background paper and provided definitions of the techniques, together with an overview of publications on recent research with NBTs (OECD, 2016). The European Food Safety Authority (EFSA) has issued two opinions concerning NBTs: the first states that their existing guidelines for risk assessment of GM plants are also appropriate for cisgenic and intragenic plants; and the second extends the guidelines also to products resulting from the use of the ZFN-3 technique (EFSA, 2012a, 2012b). However, individual national regulatory bodies in Europe have not all taken this position. The UK Advisory Committee on Releases to the Environment (ACRE) has stated that only products of cisgenesis and intragenesis should be regarded as GMOs (ACRE, 2013), while the Dutch Commission on Genetic Modification (COGEM) has gone even further, to argue that cisgenic plants should be exempt from GMO legislation since only genetic elements from the same or a cross-compatible species are introduced (COGEM, 2006, 2010). Moreover, Germany’s Central Committee on Biological Safety has classified organisms modified by means of NBTs such as ZFN and ODM as not being genetically modified (Federal Office of Consumer Protection and Food Safety [BVL], 2016), while the Swedish Board of Agriculture has concluded that products of CRISPR/Cas9 should not be subject to European GMO legislation (Umeå Plant Science Centre, 2016). Taking an opposite view, a legal analysis carried out by the German Federal Agency for Nature Conservation concluded that organisms produced using the new techniques fall within the scope of the EU’s GMO legislation (BVL, 2016), and the Environment Agency of Austria has indicated that a case-specific risk assessment and the application of precautionary principle is necessary (Eckerstorfer, Miklau, & Gaugitsch, 2014). Beyond Europe, both the US Food and Drug Administration (USDA) and Department of Agriculture (USDA) have already approved the commercial production of a potato that contains no foreign DNA and uses an RNA interference technique to reduce the level of polyphenol oxidase responsible for bruising and browning (Waltz, 2012). Further, a canola crop generated using genome editing was authorized for widespread cultivation in the United States as well as in Canada (Waltz, 2015).

1. *The CPB introduced the new term “living modified organism,” which it defines as “any living organism that possesses a novel combination of genetic material obtained through the use of modern biotechnology.” It is basically a GMO that is alive and capable of passing on its genes to a subsequent generation.*

Notably, the opinions of scientific academies have been more consistent. The European Academies Science Advisory Council (EASAC), a body of national science academies of the EU Member States, has argued that products of NBTs should not fall under GMO legislation when they do not contain foreign DNA. EASAC noted that in some cases the product cannot be distinguished from one generated by conventional techniques, and also argues that the new techniques allow much more precise and targeted changes compared with mutagenesis used in conventional breeding, where changes in the genome are induced by chemicals or radiation, creating multiple, unknown, and unintended mutations (EASAC, 2013). The view of the regulatory authorities in both the United States and Canada that the safety of new crop varieties should be assessed according to their characteristics rather than by the method with which they are produced, is shared by a number of bodies, including the UK's Biotechnology and Biological Sciences Research Council, the German Academies, the European Plant Science Organisation and the French High Council for Biotechnology (Laaninen, 2016).

According to the American Seed Trade Association (ASTA), the use of NBTs is being hampered at the research and development stage because of unclear policy and uncertain regulatory status (ASTA, 2017). The European Seed Association further argues that classifying NBTs under GMO legislation would prevent Europe's predominantly small- and medium-sized plant breeding companies from developing and using them, thus driving research away from Europe (Laaninen, 2016), noting that the European seed industry of more than 7,000 companies is a global leader in innovation (International Seed Federation [ISF], 2013) with a market value of about EUR 9 billion. Taking an opposing stance, the International Federation of Organic Agriculture Movements (IFOAM), which represents organic food producers and farming sector, argues that plants bred using the new techniques should be subject to risk assessment as well as mandatory traceability and labeling requirements, similarly as those applied to GMOs. EcoNexus, a European public interest and scientific research organization noted for its anti-GMO stance, considers that the use of NBTs should be regulated as rigorously as current GM techniques, as they might cause unintended effects with unpredictable consequences (Laaninen, 2016).

Today we are at a crossroads. NBTs have enormous potential for making changes to the genome, but do we have the appropriate tools to determine whether they are safe?

## Biosafety Concerns Raised by Specific NBTs

### *Agroinfiltration*

Agroinfiltration has been in use since the 1980s and is based on the transient expression of *Agrobacterium* genetic constructs in plant tissues. The technique is widely employed for the selection of disease-resistant plants (Vleeshouwers et al., 2008; Zenna et al., 2006) and for the experimental production of specific recombinant proteins (Gomez, Zoth, Asurmendi, Rovere, & Berinstein, 2009; Pogue et al., 2010). If applications employ RNAi constructs, then unexpected effects caused by inheritable epigenetic effects on gene regulation may result. However, such unintended methylation can also occur with other non-NBT breeding techniques, either spontaneously (Manning et al., 2006; Rhee, Sekhon, Chopra, & Kaeppeler, 2010), as a consequence of tissue culture (Krizova, Fojtova, Depicker, & Kovarik, 2009), or chemically (Fieldes, Schaeffer, Krech, & Brown, 2005). To date, however, when the technique has been employed in plant breeding selection programs, the offspring plants have been found to be free of any *Agrobacterium* genetic material (Lusser et al., 2011). Thus, as long as the GM *Agrobacterium* and its T-DNA are absent, any offspring growing from seed should not be considered GMOs (ACRE, 2013). In Argentina, it is recognized that the genetic modifications are done on mature plants and are therefore not heritable. Further, the authorities stipulate that the GMO to be released into the environment, and thus the actual subject of regulation, should be the GM microorganism (Whelan & Lema, 2015).

### *Cisgenesis and Intragenesis*

Biosafety issues from the use of cisgenesis and intragenesis have been widely discussed in the literature (De Cock Buning, Lammerts Van Bueren, Haring, De Vriend, & Struik, 2006; Durham, Doucet, & Unruh Snyder, 2011; Giddings, 2006; Myskja, 2006; Prins & Kok, 2010; Viswanath & Strauss, 2010). These techniques are usually based on the transfer of a man-made genetic construct into the plant genome. Thus, the resulting product in most cases would be considered to have incorporated a new combination of genetic material and therefore would be considered a GMO (Whelan & Lema, 2015). However, for cisgenesis—according to EFSA—a comprehensive molecular characterization is a key component of the risk assessment, but they have also stated that should the donor plant and the newly-

expressed proteins in cisgenic plants have a corresponding use and history of safe consumption as food and feed, then—depending on the particular event—lesser amounts of event-specific data may be needed for the risk assessment. Since the cisgenes were derived from the host genus gene pool and remain the same as those in the donor plant, EFSA concluded that any resultant hazards would be similar to those associated with conventionally-bred plants (EFSA, 2012a). Whether cisgenesis will lead to a biosafety concern will depend on the function and expression level of the resulting protein: increased levels of proteins and their metabolites may have a neutral effect; exhibit an adverse effect, or have a desirable consequence. This will be the same for pleiotropic effects, which can also occur during conventional breeding and transgenesis (Ge et al., 2004; Ravel et al., 2009; Thompson et al., 1999; Uauy, Brevis, & Dubcovsky, 2006). EFSA concluded that their existing guidance for the risk assessment of GM plants was applicable for the evaluation of cis- and intra-genic plants and their derived food and feed products and saw no need for further development of the guidance (EFSA, 2012a).

### **CRISPR/Cas-Nucleases**

The CRISPR/Cas9 technology is revolutionary due to its relatively low cost, easy application, flexibility, and precision. However, CRISPR/Cas systems have been known to lead to off-target effects in plants (Zhao & Wolt, 2017). Depending on the nucleotide sequence of guide RNAs, the CRISPR/Cas nucleases may trigger unintended deleterious effects on the regulation of cellular gene expression by the RNAi-system. The probability, however, of CRISPR/Cas9 methods resulting in unintended deleterious effects on the expression of other genes is low due to the precision in targeting the host sequence (Heinemann, Agapito-tenfen & Carman, 2013).

### **Grafting GM Rootstock**

It has been demonstrated that scions and their rootstock can exchange genetic material in the vicinity of the grafting site (Rusk, 2009; Stegemann, Keuthe, Greiner, & Bock, 2012), but in contrast, there are other studies—e.g., with grafted apples—where no evidence of transgene transfer into the scion was found (Smolka, Li, Heikelt, Welander, & Zhu, 2010). Further, other authors have concluded that gene transfer into the reproductive cells of the scion is unlikely (COGEM, 2006; Haroldsen, Paulino, Chi-Ham, & Bennett, 2012; Lusser et al.,

2011; Schaart & Visser, 2009). This is substantiated by a report that neither pollen nor seeds produced by the scion contain sequences of transgenic origin, therefore transgene flow into the environment may only become a biosafety concern if the GM rootstock is able to produce seedlings (COGEM, 2006). However, GMO regulators in certain jurisdictions may well require that such plants fall under their oversight; as for “conventional” GM plants, for example in Argentina, the whole plants to be commercially released are likely to be regulated as GM plants regardless of the GM part being the rootstock or scion (Whelan & Lema, 2015).

### **Oligonucleotide-directed Mutagenesis (ODM)**

ODM has the potential to lead to the formation of novel proteins and, under certain circumstances, introduce mutations with unintended undesirable effects. In many cases, it will be impossible to recognize the method used to produce the variation, as exactly the same DNA changes could be introduced using conventional, mutational breeding, or genetic modification techniques (ACRE, 2011). Furthermore, in those cases where epigenetic changes have been made, especially improvements through the loss of gene function, there will be no DNA sequence changes at all. From a process-based perspective, plants generated through ODM rely on modern biotechnology techniques listed in legal GMO definitions and thus will be GMOs. On the other hand, from a product-based perspective, it can be argued that the plants exhibit modifications in their genetic material that could also occur in a natural way; therefore, they should not be considered as GMOs (Lusser et al., 2011), with COGEM recommending that the resulting plants be excluded from regulatory oversight (COGEM, 2010).

### **Reverse Breeding**

The products of reverse plant breeding are usually either F1 hybrids or homozygous parental lines, each free of introduced DNA sequences, and as such, cannot be distinguished from their conventional counterparts (ACRE, 2011; Lusser et al., 2011). Although methylation of non-target sequences may occur during the process, leading to the alteration of the expression level of non-target genes, this can also occur in conventional plant breeding or spontaneously (Manning et al., 2006), for example during tissue-culture (Miguel & Marum, 2011) or by chemical means (Fieldes et al., 2005). With certain GM techniques, residues of genetic material from the bacteria or viruses used as shuttles for gene delivery may be left in the host plant. These foreign sequences are gener-

**Table 1. Countries where regulatory positions on plants derived from specific NBTs have been taken.**

Country	New breeding technique									Reference	
	SDN	ZFN	TALEN	CRISPR/ Cas	Reverse breeding	RdDM	Cis-/intra- genesis	Grafting	Agro- filtration		
Argentina	+/- <sup>1</sup>				cbc <sup>2</sup>	-	-	+	+	+	Whelan and Lema (2015)
Australia & New Zealand	+/- <sup>1</sup>				-	-		+	+	-	FSANZ (2013)
Canada	Regulations not process-based, thus final product only regulated if it is novel to Canada.									OECD (2016)	
China	Producing plants using CRISPR/Cas but no information found concerning possible regulation.										
European Union	+						+				Lusser et al. (2011), Lusser et al. (2012), Laaninen (2016)
India	Case-by-case review. GMO requires a transgene. Review of NBTs passed to states.										
Japan	No known regulations. Special taskforce created to discuss importance of NBTs for seed industry.										
Russia	Federal Law 358FZ effectively bans all activities with GMOs in Russian Federation since 2016. No known discussions concerning NBT regulations.										
United States				-	-						Schuttelaar and Partners (2015)

<sup>1</sup>+ for SDN3 - for SDN1 & SDN2 <sup>2</sup> case-by-case

ally easily detectable in the resultant product; however, with certain NBTs such as reverse breeding, it is possible to remove the segments of genetic material derived from shuttle organisms, and even to modify them without inserting foreign gene sequences. The resultant plants therefore present a conundrum for those regulatory systems that are process-based, and as yet there has been little—if any—positions taken by such regulatory authorities. Neither ACRE nor COGEM see any justification for suggesting that plants generated by this approach are GMOs (ACRE, 2013), while the Argentine authorities will require evidence that the transgene has actually been removed (Whelan & Lema, 2015).

### **RNA-directed DNA Methylation (RdDM)**

Current analytical detection methods cannot differentiate between an RdDM-induced methylation pattern and native methylation patterns, therefore plants cannot be clearly identified as being modified by RdDM (Schaart & Visser, 2009). It is also acknowledged that although epigenetic variations can occur as a result of RdDM, they can also occur in any plant resulting from plant breeding which has undergone an *in vitro* tissue culture phase (Bairu, Aremu, & Staden, 2011). Thus the same conundrum for process-based regulatory systems arises as to whether and how these products may be regulated.

### **Zinc-finger Nucleases (ZFN) and Transcription Activator-like Nucleases (TALEN)**

A risk of off-target cleavage and cytotoxicity when zinc-finger domains are fused with Fok1 nuclease has been reported from the application of ZFNs, with the off-target cleavage attributed to non-specific DNA binding of the zinc-finger domains by Fok1 nuclease (De Francesco, 2012). Similarly, TALENs also employ Fok I nuclease, but owing to their longer DNA recognition sites, TALENs are claimed to be more specific than ZFNs, with fewer unwanted off-target effects (Li, Liu, Spalding, Weeks, & Yang, 2012). However, these processes also occur during conventional mutagenesis (Lusser et al., 2011). Therefore it can be argued that the products of ZFN and TALEN techniques should be treated similarly. The regulatory status of ZFN and TALEN remains under consideration in many countries around the world (Gruskin, 2012).

### **Discussion**

The biosafety issues concerning GMOs have been questioned and discussed minutely over the last 30 years, whereas discussion of the issues surrounding NBTs has increased only recently. In many countries, crops developed through GMO techniques are subject to mandatory regulatory oversight while plant varieties obtained through conventional breeding methods are marketed without specific safety evaluation, except for their distinctness, uniformity, and stability (DUS). In the era of

new techniques, the first question that arises is whether the crops generated through NBTs should, in fact, fall under current GMO regulatory systems, and, if they must, the second question is whether they present additional challenges for which the systems need to be adjusted. The reluctance of several countries to take a definitive position on NBTs is probably related to the political and economic effects of NBT approval that are difficult to predict, as agricultural biotechnology is a sensitive issue in many countries, and international trade relations can be affected by the position of the governments concerned. The expected difficulties in detecting NBT products—and differentiating them from products obtained through more conventional breeding methods—exacerbate the situation. Further, if a country exempts certain NBTs from regulation, trading partner countries with a different view on NBTs may regard such imports as unacceptable, and—in a worst-case scenario—this might lead to blocking the overall trade. This is, however, a matter that needs to be addressed at the global level by initiatives governing international trade, such as the World Trade Organization.

Those regulatory systems based on process, rather than product, have generally concluded that, in principle, the protocols used for the assessment of GMOs would also be appropriate to address the risks of those NBTs that will fall under their purview. The main question that needs to be addressed is whether the crops generated using NBTs differ from existing crops and how their products will be classified for regulatory purposes under the current definitions of genetic modification. The basic principles implemented in biosafety regulatory frameworks such as the European Commission Directives; the Cartagena Protocol on Biosafety; the Canadian Plants with Novel Traits Regulation; and the US joint directives from the USDA, US Food and Drug Administration (FDA), and US Environmental Protection Agency (EPA) are all good starting points for taking into account certain NBTs. As with most conventional plant breeding techniques, NBTs have the potential to introduce unintended effects and the impacts of any that are deleterious should be evaluated in those jurisdictions that require such regulatory oversight.

NBTs are very diverse in their approaches and methodology and they can be used either alone in the breeding of a certain crop or in combination with other NBTs, with conventional breeding approaches, or with other GMO technologies. Therefore, a critical question concerns whether their products will fall under regulatory oversight. NBTs may offer many advantages over current GM technologies, and if they are exempt from

GMO regulation, small enterprises and academic institutions will benefit most from a faster and cheaper road to commercialization.

NBTs offer great technical advantages for producing genetic variation, targeted mutagenesis, targeted introduction of new genes, and gene silencing and fast-track screening selection in breeding programs. Stakeholders and seed companies have raised concerns that unnecessary regulation of products derived from NBTs will result in costly regulatory responsibilities, stifle innovation, and prevent the adoption of innovative breeding applications. The regulatory uncertainty of NBTs is a challenge for developers because registration costs will be low if the products from NBTs are to be classified as non-GMO, whereas the costs will be substantially higher if classified as GMO. The regulatory status of NBTs will affect the decisions of the developers on whether to use these techniques only for the improvement of traits in products with very high value or for a large portfolio of crop applications. It is important therefore that governments avoid the creation of trade barriers due to incompatible and non-harmonious regulatory practices and policies.

## References

- Advisory Committee on Releases to the Environment (ACRE). (2011). *Advice on a plant breeding technique involving oligo-directed mutagenesis: RTDS™*. London: Author.
- ACRE. (2013). ACRE advice: New techniques used in plant breeding. London: Author. Available on the World Wide Web: [https://www.gov.uk/government/uploads/system/uploads/attachment\\_data/file/239542/new-techniques-used-in-plant-breeding.pdf](https://www.gov.uk/government/uploads/system/uploads/attachment_data/file/239542/new-techniques-used-in-plant-breeding.pdf).
- American Seed Trade Association (ASTA). (2017). *Promoting innovation in seed and trade development*. Alexandria, VA: Author. Available on the World Wide Web: <http://www.amseed.org/pdfs/issues/innovation-and-policy/promoting-innovation-in-seed-and-crop-development.pdf>.
- Bairu, M.W., Aremu, A.O., & Staden, J.V. (2011). Somaclonal variation in plants: Causes and detection methods. *Plant Growth Regulation*, 63, 147-173.
- Federal Office of Consumer Protection and Food Safety (BVL). (2016). *Opinion on the legal classification of new plant breeding techniques, in particular ODM and CRISPR-Cas9*. Braunschweig, Germany: Author. Available on the World Wide Web: [https://www.bvl.bund.de/SharedDocs/Downloads/06\\_Gentechnik/Opinion\\_on\\_the\\_legal\\_classification\\_of\\_New\\_Plant\\_Breeding\\_Techniques.pdf?\\_\\_blob=publication-File&v=2](https://www.bvl.bund.de/SharedDocs/Downloads/06_Gentechnik/Opinion_on_the_legal_classification_of_New_Plant_Breeding_Techniques.pdf?__blob=publication-File&v=2).
- Bortesi, L., & Fischer R., (2015). The CRISPR/Cas9 system for plant genome editing and beyond. *Biotechnology Advances*, 33, 41-45.

- Dutch Commission on Genetic Modification (COGEM). (2006). *New techniques in plant biotechnology* (Report CGM/061024-02). Bilthoven. The Netherlands: Author.
- COGEM. (2010). *The status of oligonucleotides within the context of site-directed mutagenesis* (Advice Report CGM/100701-03). Bilthoven. The Netherlands: Author.
- Cools, H.J., & Hammond-Kosack, K.E. (2013). Exploitation of genomics in fungicide research: Current status and future perspectives. *Molecular Plant Pathology*, 14, 197-210.
- De Cock Buning, T., Lammerts Van Bueren, E.T., Haring, M.A., De Vriend, H.C., & Struik, P.C. (2006). 'Cisgenic' as a product designation. *Nature Biotechnology*, 24, 1329-1331.
- De Francesco, L. (2012). Tal effector DNA structure. *Nature Biotechnology*, 30, 158.
- Durham, T., Doucet, J., & Unruh Snyder, L. (2011). Risk of regulation or regulation of risk? A de minimus framework for genetically modified crops. *AgBioForum*, 14, 61-70. Available on the World Wide Web: <http://www.agbioforum.org>.
- Eckerstorfer, M., Miklau, M., & Gaugitsch, H. (2014). *New plant breeding techniques and risks associated with their application* (Report REP-0477). Vienna: Umweltbundesamt GmbH.
- Edwards, D., Batley, J., & Snowdon, R.J. (2012). Accessing complex crop genomes with next-generation sequencing. *Theoretical Applied Genetics*, 126(1), 1-11.
- European Academies Science Advisory Council (EASAC). (2004). *Genomics and crop plant science in Europe* (EASAC Policy Report 02). Brussels: Author.
- EASAC. (2011). *Plant genetic resources for food and agriculture: Roles and research priorities in the European Union* (EASAC Policy Report 17). Brussels: Author.
- EASAC. (2013). *Planting the Future: Opportunities and Challenges for Using Crop Genetic Improvement Technologies for Sustainable Agriculture*. EASAC Report 21. Brussels: Author.
- European Food Safety Authority (EFSA). (2012a). Scientific opinion addressing the safety assessment of plants developed through cisgenesis and intragenesis. *EFSA Journal*, 10(2), 2561.
- EFSA. (2012b). Scientific opinion addressing the safety assessment of plants developed using zinc finger nuclease 3 and other site-directed nucleases with similar function. *EFSA Journal*, 10(10), 2943.
- Fears, R., Aro, E.-M., Pais, M.S., & ter Meulen, V. (2014). How should we tackle the global risks to plant health? *Trends in Plant Science*, 19(4), 206-208.
- Fernie, S. (2009). Metabolomics-assisted breeding: A viable option for crop improvement? *Trends in Genetics*, 25, 39-48.
- Fieldes, M.A., Schaeffer, S.M., Krech, M.J., & Brown, J.C.L. (2005). DNA hypomethylation in 5-azacytidine-induced early-flowering lines of flax. *Theoretical and Applied Genetics*, 111, 136-149.
- Food and Agriculture Organization of United Nations (FAO). (2009). *How to feed the world in 2050*. Rome: Author. Available on the World Wide Web: [http://www.fao.org/fileadmin/templates/wsfs/docs/expert\\_paper/How\\_to\\_Feed\\_the\\_World\\_in\\_2050.pdf](http://www.fao.org/fileadmin/templates/wsfs/docs/expert_paper/How_to_Feed_the_World_in_2050.pdf).
- FAO. (2015). Food security; Population; Inputs. In *FAOSTAT statistical pocketbook: World food and agriculture*. Rome: Author. Available on the World Wide Web: <http://www.fao.org/3/a-i4691e.pdf>.
- Food Standards Australia New Zealand (FSANZ). (2013). *New plant breeding techniques: Report of a workshop hosted by Food Standards Australia New Zealand*. Kingston, Australia & Wellington, New Zealand: Author. Available on the World Wide Web: <http://www.foodstandards.gov.au/publications/Documents/New%20Plant%20Breeding%20Techniques%20Workshop%20Report.pdf>.
- Ge, L., Chen, H., Jiang, J.F., Zhao, Y., Xu, M.L., Xu, Y.Y., et al. (2004). Overexpression of OsRAA1 causes pleiotropic phenotypes in transgenic rice plants, including altered leaf, flower and root development and root response to gravity. *Plant Physiology*, 135, 1502-1513.
- Giddings, L. (2006). 'Cisgenic' as a product designation. *Nature Biotechnology*, 24, 1329.
- Gomez, E., Zoth, S.C., Asurmendi, S., Rovere, C.V., & Berinstein, A. (2009). Expression of Hemagglutinin-Neuraminidase glycoprotein of Newcastle Disease Virus in agroinfiltrated *Nicotiana benthamiana* plants. *Journal of Biotechnology*, 144, 337-340.
- Gruskin, D. (2012). Agbiotech 2.0. *Nature Biotechnology*, 30, 211-214.
- Haroldsen, V.M., Paulino, G., Chi-Ham, C.L., & Bennett, A.B. (2012). Regulatory status of transgranted plants is unclear. *California Agriculture*, 66, 68-69.
- Heinemann, J., Agapito-tenfen, S., & Carman, J. (2013). A comparative evaluation of the regulation of GM crops or products containing dsRNA and suggested improvements to risk assessment. *Environment International*, 55, 43-55.
- International Seed Federation (ISF). (2013). *Estimated value of the domestic seed market in selected countries for the year 2012*. Nyon, Switzerland: Author.
- James, C. (2016). *Global status of commercialized biotech/GM crops* (ISAAA Brief No. 52-2016). Ithaca, NY: ISAAA.
- Jander, G., Baerson, S.R., Hudak, J.A., Gonzalez, K.A., Gruys, K., Gomez, E., et al. (2003). Ethylmethanesulfonate saturation mutagenesis in Arabidopsis to determine frequency of herbicide resistance. *Plant Physiology*, 131(1), 139-146.
- Jiang, W., Zhou, H., Bi, H., Fromm, M., Yang, B., & Weeks, D.P. (2013). Demonstration of CRISPR/Cas9/sgRNA-mediated targeted gene modification in Arabidopsis, tobacco, sorghum and rice. *Nucleic Acids Research*, 41, 188.
- Jiao, Y., Tausta, S.L., Gandotra, N., Sun, N., Liu, T., Clay, N.K., Ceserani, T., Chen, M., Ma, L., Holford, M., Zhang, H.Y., Zhao, H., Deng, X.W., & Nelson, T. (2009). A transcriptome atlas of rice cell types uncovers cellular, functional and developmental hierarchies. *Nature Genetics*, 41, 258-263.

- Jones, H.D. (2015). Regulatory uncertainty over genome editing. *Nature Plants*, 8(1), 14011.
- Jorin-Novo, J.V., Maldonado, A.M., Echevarria-Zomeno, S., Valledor, L., Castillejo, M.A., & Curto, M. (2009). Plant proteomics update (2007-2008): Second-generation proteomic techniques, an appropriate experimental design, and data analysis to fulfil MIAPE standards, increase plant proteome coverage and expand biological knowledge. *Journal of Proteomics*, 72, 285-314.
- Krizova, K., Fojtova, M., Depicker, A., & Kovarik, A. (2009). Cell culture-induced gradual and frequent epigenetic reprogramming of invertedly repeated tobacco transgene epialleles. *Plant Physiology*, 149, 1493-1504.
- Laaninen, T. (2016, May). *New plant-breeding techniques: Applicability of GM rules* (Briefing). Brussels: European Parliamentary Research Service (EPRS). Available on the World Wide Web: [http://www.europarl.europa.eu/RegData/etudes/BRIE/2016/582018/EPRS\\_BRI\(2016\)582018\\_EN.pdf](http://www.europarl.europa.eu/RegData/etudes/BRIE/2016/582018/EPRS_BRI(2016)582018_EN.pdf).
- Lema, M. (2017). Regulatory challenges: Technology-based vs. product-based regulations and potential impact on product monitoring. Poster presented at the *International Symposium on the Biosafety of Genetically Modified Organisms (ISBGM014)*, June 4-8, 2017, Guadalajara, Mexico.
- Li, T., Liu, B., Spalding, M.H., Weeks, D., & Yang, B. (2012). High-efficiency TALEN based gene editing produces disease-resistant rice. *Nature Biotechnology*, 30, 390-392.
- Lusser, M., Parisi, C., Plan, D., & Rodríguez-Cerezo, E. (2011). *New plant breeding techniques: State-of-the-art and prospects for commercial development* (JRC Report EUR 24760 EN). Seville, Spain: European Commission Joint Research Centre (JRC). Available on the World Wide Web: <http://ftp.jrc.es/EURdoc/JRC63971.pdf>.
- Lusser, M., Parisi, C., Plan, D., & Rodriguez-Cerezo, E. (2012). Deployment of new technologies in plant breeding. *Nature Biotechnology*, 30, 231-239.
- Manning, K., Tor, M., Poole, M., Hong, Y., Thompson, A.J., Giovannoni, J.J., & Seymour, G.B. (2006). A naturally occurring epigenetic mutation in a gene encoding an SBP-box transcription factor inhibits tomato fruit ripening. *Nature Genetics*, 38, 948-952.
- Miguel, C., & Marum, L. (2011). An epigenetic view of plant cells cultured *in vitro*: Somaclonal variation and beyond. *Journal of Experimental Botany*, 62, 3713-3725.
- Morrell, P.L., Buckler, E.S., & Ross-Ibarra, J. (2012). Crop genomics: Advances and applications. *Nature Review Genetics*, 13, 85-96.
- Myskja, B.K. (2006). The moral difference between intragenic and transgenic modification of plants. *Journal of Agricultural and Environmental Ethics*, 19, 225-238.
- Organization for Economic Cooperation and Development (OECD). (2016). *Environmental risk assessment for products derived from new plant breeding techniques*. Paris: Author.
- Pogue, G.P., Vojdani, F., Palmer, K.E., Hiatt, E., Hume, S., Phelps, J., et al. (2010). Production of pharmaceutical-grade recombinant aprotinin and a monoclonal antibody product using plant-based transient expression systems. *Plant Biotechnology Journal*, 8(5), 638-654.
- Prins, T.W., & Kok, E.J. (2010, April). *Food and feed safety aspects of cisgenic crop plant varieties* (Report 2010.001). Wageningen, The Netherlands: Wageningen University & Research Centre, RIKILT-Institute of Food Safety. Available on the World Wide Web: <http://edepot.wur.nl/157733>.
- Ravel, C., Martre, P., Romeuf, I., Dardevet, M., El-Malki, R., Bordes, J., et al. (2009). Nucleotide polymorphism in the wheat transcriptional activator spa influences its pattern of expression and has pleiotropic effects on grain protein composition, dough viscoelasticity, and grain hardness. *Plant Physiology*, 151, 2133-2144.
- Rhee, Y., Sekhon, R.S., Chopra, S., & Kaeppeler, S. (2010). Tissue culture-induced novel epialleles of a myb transcription factor encoded by pericarp color1 in Maize. *Genetics*, 186(3), 843-855.
- Rusk, N. (2009). Grafting as a potent molecular tool. *Nature Methods*, 6, 484.
- Schaart, J.G., & Visser, R.G.F. (2009). *Novel plant breeding techniques—Consequences of new genetic modification-based plant breeding techniques in comparison to conventional plant breeding* (Report 2009-02, pp. 2785-2794). Bilthoven: The Netherlands Commission on Genetic Modification (COGEM).
- Schuttelaar & Partners. (2015, June). *The regulatory status of new breeding techniques in countries outside the European Union*. The Hague, The Netherlands: Author. Available on the World Wide Web: <http://www.nbtplatform.org/background-documents/rep-regulatory-status-of-nbts-ousside-the-eu-june-2015.pdf>.
- Secretariat of the Convention on Biological Diversity. (2000). The Cartagena Protocol on biosafety to the convention on biological diversity: Text and annexes. Montreal: Author. Available on the World Wide Web: <http://www.cbd.int/doc/legal/cartagena-protocol-en.pdf>.
- Smolka, A., Li, X.Y., Heikelt, C., Welander, M., & Zhu, L.-H. (2010). Effects of transgenic rootstocks on growth and development of non-transgenic scion cultivars in apple. *Transgenic Research*, 19, 933-948.
- Stegemann, S., Keuthe, M., Greiner, S., & Bock, R. (2012). Horizontal transfer of chloroplast genomes between plant species. *Proceedings of the National Academy of Sciences USA*, 109, 2434-2438.
- Thompson, A., Barry, C., Jarvis, M., Vrebalov, J., Giovannoni, J., Grierson, D., & Seymour, G. (1999). Molecular and genetic characterization of a novel pleiotropic tomato-ripening mutant. *Plant Physiology*, 120, 383-389.
- Till, B.J., Cooper, J., Tai, T.H., Colowit, P., Greene, E.A., Henikoff, S., & Comai, L. (2007). Discovery of chemically

- induced mutations in rice by TILLING. *BMC Plant Biology*, 7, 19.
- Uauy, C., Brevis, J.C., & Dubcovsky, J. (2006). The high grain protein content gene Gpc-B1 accelerates senescence and has pleiotropic effects on protein content in wheat. *Journal of Experimental Botany*, 57, 2785-2794.
- Umeå Plant Science Centre. (2016). "Green light in the tunnel"! Swedish Board of Agriculture: A CRISPR-Cas9-mutant but not a GMO (Press Release). Umeå, Sweden: Author. Available on the World Wide Web: <http://www.upsc.se/about-upsc/news/4815-green-light-in-the-tunnel-swedish-board-of-agriculture-a-crispr-cas9-mutant-but-not-a-gmo.html>.
- Viswanath, V., & Strauss, S.H. (2010, September). *Modifying plant growth the cisgenic way* (ISB News Report). Blacksburg, VA: Virginia Tech, Information Systems for Biotechnology (ISB).
- Vleeshouwers, V.G.A.A., Rietman, H., Krenek, P., Champouret, N., Young, S.K., Wang, M., et al. (2008). Effector genomics accelerates discovery and functional profiling of potato disease resistance and *Phytophthora infestans* avirulence genes. *Plos One*, 3(8), 2875.
- Whelan, A., & Lema, M. (2015). Regulatory framework for gene editing and other new breeding techniques (NBTs) in Argentina. *GM Crops & Food*, 6(4), 253-265.
- Waltz, E. (2012). Tiptoeing around transgenics. *Nature Biotechnology*, 30, 215-217.
- Waltz, E. (2015). USDA approves next-generation GM potato. *Nature Biotechnology*, 33(1), 12-13.
- Wang, Y., Cheng, X., Shan, Q., Zhang, Y., Liu, J., Gao, C., & Qiu, J.L. (2014). Simultaneous editing of three homoeoalleles in hexaploid bread wheat confers heritable resistance to powdery mildew. *Nature Biotechnology*, 32, 947-951.
- Zenna, N., Sta Cruz, F., Javier, E., Duka, I., Barrion, A., & Azzam, O. (2006). Genetic analysis of tolerance to rice tungro bacilliform virus in rice (*Oryza sativa* L.) through agroinoculation. *Journal of Phytopathology*, 154(4), 197-203.
- Zhao, H., & Wolt, J.D. (2017). Risk associated with off-target plant genome editing and methods for its limitation. *Emerging Topics in Life Sciences*, 1(2), 231-240.

## Acknowledgements

The authors would like to thank Lawrence Banks for his critical review and helpful comments. The opinions presented in this manuscript do not necessarily reflect positions or policies of the International Centre for Genetic Engineering and Biotechnology (ICGEB).

---

## Box 1. New breeding techniques

Over recent years, a new generation of plant breeding techniques has emerged as a result of increased knowledge on plant genomes and molecular biology. The OECD paper described seven of these techniques generally considered as new plant breeding techniques (NBTs). Some NBTs induce site-specific genome alterations at defined sites in the genome (SDN and ODM), whereas others introduce beneficial traits through epigenetic modifications (RdDM) or modifications with genes derived from cross-compatible species (cisgenesis and intragenesis). Also techniques have emerged in which transgenes are only expressed in certain plant tissues (grafting and agro-infiltration).

### Site-directed Nucleases (SDN)

SDN refer to protein complexes that are responsible for the targeted mutagenesis of genes or the targeted insertions or deletions of the genetic material. The nuclease domain of SDN complexes can induce a site-specific double strand break in the DNA, where both complementary strands of DNA are broken. Different types of SDNs exist:

- **Zinc-finger nucleases (ZFN)** are artificial protein complexes that consist of a nuclease domain coupled to a zinc finger domain. A zinc finger is a protein motif that interacts specifically with three base pairs of DNA. Different zinc fingers target specific nucleotide triplets, and by fusing multiple zinc fingers together a specific sequence can be targeted. The nuclease that is coupled to the zinc finger complex can subsequently induce a DSB on the targeted site in the genome.
- Via a mechanism similar to ZFN, **transcription activator-like nucleases (TALENs)** can induce site-specific mutations in a genome. TALENs are artificial protein complexes in which a nuclease is coupled to a Transcription Activator-Like (TAL) effector domain. The TAL effector is a protein complex that is able to recognize and bind specific genetic sequences. TAL effectors can be engineered to target specific genetic sequences and induce mutations via the attached nuclease domain.

(Continued on next page)

### **Box 1. Continued**

- **CRISPR/Cas nucleases** are nuclease complexes. The bacterial nuclease Cas9 is a component of the adaptive immunity system in bacteria, organized to recognize and destroy foreign DNA. CRISPR/Cas nucleases are guided to a certain genomic DNA sequence by guide RNAs attached to the nuclease. The guide RNAs navigate the nuclease activity to target sequences in the crop genome, which are complementary to the recognition sequence of the guide RNA. As a result, a number of target sequences and different genome sites can be specifically targeted.

#### **Oligonucleotide-directed mutagenesis (ODM)**

ODM is a technique in which small, specific mutations are induced through synthetic oligonucleotides. ODM is currently in an early phase of commercialization.

#### **Cisgenesis and intragenesis**

With cisgenesis and intragenesis only genes that are derived from cross-compatible species are transferred into a plant genome, so the approach does not exceed natural genetic boundaries.

#### **RNA-directed DNA methylation (RdDM)**

RdDM is a technique that allows the epigenetic silencing of a targeted gene in a plant genome. This is achieved via a complex mechanism in which DNA-methylation induces chromosomal rearrangements in the promoter region of the targeted gene making it inaccessible to enzymes involved in transcription. As far as current publications show, RdDM is still in the research phase and no commercial products have yet been developed. A current major challenge in RdDM is to achieve stable gene silencing over multiple generations.

#### **Reverse breeding**

Reverse breeding is a technique in which RNA interference is used to suppress meiotic recombination so that homozygous parental plants can be obtained from selected heterozygous individuals with beneficial traits. Reverse breeding is currently still in an early phase of development: only a few papers have been published so far.

#### **Grafting**

Grafting on transgenic rootstocks is mainly done with fruit crops and woody species (e.g. plum, grape, tomato, walnut). The rationale behind this approach is that the transformed rootstock can confer an enhanced genetic characteristic (e.g. resistance traits) without the presence of transgenic DNA in the fruit or nut.

#### **Agro-infiltration**

Agro-infiltration allows rapid, transient high level expression of transgenes in defined plant tissues through transformation by *A. tumefaciens*. Agro-infiltration is reported mainly as an approach for producing pharmaceutical proteins and as a system to test genes with unknown function.