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New Techniques in Agricultural Biotechnology

High Level Group of Scientific Advisors

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High Level Group of Scientific Advisors

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1 Current HLG members are listed in the Commission's Register of Expert Groups, http://ec.europa.eu/transparency/regexpert/

2 HLG Chair and Member of HLG until 28 February 2017.

3 SAPEA brings together the outstanding knowledge and expertise of Fellows from over 100 Academies and Learned Societies in over 40 countries across Europe. Funded through the EU's Horizon 2020 programme, the SAPEA consortium comprises Academia Europaea (AE), All European Academies (ALLEA), the European Academies Science Advisory Council (EASAC), the European Council of Academies of Applied Sciences, Technologies and Engineering (Euro-CASE) and the Federation of European Academies of Medicine (FEAM).

Introduction

1. INTRODUCTION

A variety of techniques are available to select and introduce desirable traits[4] in animals, plants, and microorganisms[5] used for food and feed production. These range from conventional breeding techniques (CBT), established techniques of genetic modification (ETGM) and a growing number of what are commonly called new breeding techniques (NBT). The NBT can be used in combination with CBT and ETGM, and all techniques remain in use in parallel to a greater or lesser degree.

The NBT build on recent advances in biotechnology and molecular biology, and the sequencing and annotation of genomes of a variety of species. As with ETGM and some CBT, the application of these NBT is the subject of debate.

This Note is intended to inform the reader about the nature and characteristics of NBT and how they are similar to, and different from, CBT and ETGM. It describes the most important examples of CBT, ETGM, and NBT in the context of their direct agricultural application in plants, animals, and microorganisms for the production of food and feed (such examples are however by definition non-exhaustive). The Note also briefly outlines the agricultural application of NBT in the fields of synthetic biology and gene drive.

The 'scoping paper' in Annex 1 provides the basis for these groupings of techniques and the definitions of some terms, but for illustrative purposes: CBT includes for example, simple selection, sexual crosses, mutation breeding, *etc.*; ETGM refers to the production of transgenic organisms; and NBT to the wide range of techniques including genome editing (*e.g.* with CRISPR-Cas systems), epigenetic modification, *etc*.

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4 Important terms which may not be familiar to the reader are defined in the glossary.

5 Microorganisms most relevant for agriculture encompass both bacteria and yeast (microscopic fungi). Macroscopic fungi (mushrooms) are not excluded from consideration but are not systematically mentioned for ease of reading and due to the comparatively greater application of these techniques in plants, animals and microorganisms.

The Note also compares these NBT, where relevant, with ETGM and CBT according to a number of criteria including: the detectability and identification of their products; the speed with which the desired outcome can be achieved and its cost; and the degree of maturity of the technique (that is, whether still in development in the laboratory or ready for use in agricultural contexts, for example field trials for plants). Aspects related to safety are also briefly discussed.

The various techniques are compared from a scientific and technical perspective. Consequently, terms are used according to their scientific rather than legal meaning. As indicated above, however, the grouping of techniques into categories is based in part on legal/regulatory definitions included in the 'scoping paper'.

In addition to its general policy to consider as evidence only information which is in the public domain at the time of publication of scientific advice, and in view of the large amount of information available, the HLG has preferentially referred to published reviews of the literature, scientific reports and existing published opinions from recognised scientific or science-based organisations in the following.

Summary

2. SUMMARY

This is a summary of the main statements made in the accompanying explanatory note on New Techniques in Agricultural Biotechnology (henceforth, the 'Note'). It also serves as a reader's guide to the more detailed Note itself.

The Note is presented in response to a request, formulated in the corresponding Scoping Paper (adopted by the HLG, 25 November 2016, Annex 1), by the European Commissioners for Health and Food Safety, Vytenis Andriukaitis; and for Science, Research and Innovation, Carlos Moedas; to the Scientific Advice Mechanism's High Level Group of Scientific Advisors (HLG). The HLG was asked to provide:

> *1) "…an up-to-date overview on new techniques in agricultural biotechnology {…}, the key characteristics of each of these (such as underlying molecular mechanisms and products obtained)… {and to} describe potential agricultural applications of {these} new techniques in the field of synthetic biology and gene drives…"* and;
>
> *2) "…to explain the differences and similarities of each new technique as compared to i) established techniques of genetic modification and ii) conventional breeding techniques {according and where possible to the criteria of} safety for health and the environment, possibilities for the detection of the respective products, speed and cost to achieve the expected result and degree of maturity for field applications…".*

The Scoping Paper also specifies that "*explanations should be in scientific terms and should not examine legal issues*" and that the Note "*will be based on published literature reviews, scientific reports and existing published opinions*".

This Note is unique in that it provides a scientific comparison of the full spectrum of breeding techniques applied in agriculture according to the above set of defined criteria. The Note is *explanatory* and so does not take a position or make recommendations to policy makers with respect to the techniques under discussion.

The main statements made in the Note are summarised below. Illustrative examples assisting understanding of technical concepts and highlighting key comparisons are also provided, but a certain level of technical knowledge is assumed for reasons of brevity. The reader is therefore advised to refer to the Note (and glossary) for an explanation of techniques, terms and for the detailed comparison of techniques (in particular, tables 1A/B to 7A/B); as well as for references substantiating these statements and an explanation of the process used to develop them. Guidance on the structure of the Note, intended to aid reading, is provided at the end of this summary.

Genetic diversity, change and an introduction to the comparison of breeding techniques

- All living organisms are subject to alterations to their genetic information due to molecular processes (*e.g.* errors in genome replication, or mutations) which can occur spontaneously and due to exposure to environmental stressors. Changes that occur in individual organisms lead to the genetic diversification of populations.

- All breeding techniques applicable in agriculture (grouped[6] as requested for the purposes of the Note as conventional breeding techniques, CBT; established techniques of genetic modification, ETGM; and new breeding techniques, NBT) make use of genetic diversity and change whether naturally occurring or resulting from human intervention, in order to select or generate plants, animals or microorganisms that exhibit preferred characteristics.

- There is heterogeneity within the NBT, and some similarities between some NBT and some CBT and some ETGM, and this is reflected in the variety of end products which can result from the employment of NBT. These similarities and differences relate to 1) molecular mechanisms; 2) the size, location and frequency of the resulting genetic changes (precise and intended vs. imprecise and unintended); 3) the extent to which ETGM are employed in NBT; and 4) the presence or otherwise of exogenous[7] nucleic acids[8] in intermediate and end-products. These factors affect among others the extent to which the genetic changes are detectable.

- The genome editing subset of NBT can produce precisely located alterations to DNA sequences, ranging from 'point mutations' (changes of one or a few nucleotides, which may be either random or specified) to the insertion of (endogenous or exogenous) genes. Other NBT, such as RNA-

[6] Refer to the 'scoping paper' in Annex 1 for a list of the techniques within each group, and for the basis of these groupings.

[7] DNA or RNA originating outside the respective organism.

[8] Most commonly DNA, but also RNA in the case of CRISPR-Cas.

dependent DNA methylation (RdDM) make no changes to DNA sequences at all.

- The end products of NBT may or may not contain exogenous DNA depending largely on the technique(s) employed. The development of an end product that involves the use of NBT may additionally use ETGM in one or more intermediate steps (*e.g.* in genome editing, RdDM), agro-infiltration, *etc.*), and as a consequence, exogenous nucleic acids may be present in intermediate products but not necessarily in the end product.

- This variety and versatility of NBT explains why comparisons between NBT and CBT, and NBT and ETGM, in the Note are only made where relevant, and suggests that grouping techniques together as NBT may not be optimal for scientific or other reasons.

Safety, precision and unintended effects

- Differences between the groups of techniques (CBT, ETGM, and NBT), of relevance to unintended effects and efficiency, depend on the extent to which changes can be targeted, and how precisely they can be made. Changes made with CBT, in particular by mutation breeding in plants, require the screening of a large population in which changes have been randomly induced[9] and the selection of desirable progeny[10]. ETGM and NBT[11] by contrast do not require such extensive screening as pre-defined changes are made to defined genetic sequences or to gene expression.

- ETGM and NBT differ in the extent to which they produce 'unintended effects'. Unintended effects are, as the term suggests, effects other than those which are desired, resulting from the employment of a technique[12]. These effects can include, for example, the disruption of genes unrelated to the desired effect due to the insertion of genetic material at

[9] This resembles the natural process of genetic diversification, but at vastly higher rates.

[10] Screening and further breeding does not necessarily exclude the presence of unidentified mutations in the end product, however.

[11] In plants in particular, the immediate, modified product is usually not the end product but is used as a progenitor for further crosses.

[12] In this sense, the vast majority of effects which result from the employment of the CBT of mutation breeding are unintended effects, and desirable traits subsequently selected.

random locations in the target genome. Unintended mutations do not however always have phenotypic effects, and not all phenotypic effects are detrimental.

- Random insertion of nucleic acids is characteristic of the employment of ETGM in plants and animals[13], and multiple insertion events can also occur at untargeted and therefore uncontrolled genetic locations. By contrast, the NBT of genome editing offer not only the ability to target insertions (resulting in comparatively fewer unintended effects on the expression of other genes or their disruption) but also the ability to make small, precise and specific changes, such as point mutations[14], which can also be observed in nature. The employment of the NBT of gene editing does not exclude 'off-target' effects, where a precise change is made to a genetic sequence identical or similar to that in which the change is desired, but in another location. By contrast with unintended effects resulting from ETGM and CBT, NBT off-target effects are rare, and in general, the frequency of unintended effects in NBT products is much lower than in products of CBT and ETGM.

- The precision available from the employment of NBT and efficiency of their use means that some products can only be realistically obtained with the use of these techniques and not through the use of CBT or ETGM. The issues of unintended effects due to NBT (and in particular, genome editing related off-target effects) are the subject of much research at present as evidenced by the rapidly growing number of publications in the field.

- Conclusions cannot be drawn about the absolute or comparative safety of techniques based on the predicted occurrence of unintended effects. An assessment of safety can only realistically be made on a case-by-case basis and depends on features of the end product including:

[13] Though not in microorganisms, where due to differences in the ability to promote homologous recombination involving cellular recombination and repair mechanisms, insertion with ETGM is usually targeted and thus not random. Furthermore, in microorganisms exogenous nucleic acid can be present in a plasmid, and is maintained, without integration in the host genome.

[14] A point mutation is a single nucleotide base substitution, insertion or deletion which may nevertheless have a large phenotypic effect.

unintended and intended effects, the species, the environment in which the product is used, the agricultural practice in question, the intended use and the exposure. It is not within the scope of the Note to assess the risks presented by individual end products. Furthermore, the Note observes that genetically and phenotypically similar products deriving from the use of different techniques are not expected to present significantly different risks.

- Regardless of the technique used, the introduction of changes to genetic sequences and gene expression in an organism can induce unintended effects in the organism.

Detecting changes and identifying their causes

- Detection describes the ability to observe changes in a genome (or at a phenotypic level) whereas identification describes the ability to identify whether changes result from spontaneous mutation or technical intervention, and if so, which technique is employed. The detection of changes made with any technique in plants, animals, or microorganisms is possible with a variety of analytical methods, if detailed molecular information on the changes is available *a priori*. By contrast, without any prior information, changes introduced with any technique are difficult to detect and identification of the underlying technique is generally impossible with current analytical methods.

- Both ETGM and NBT can involve the introduction of exogenous nucleic acids in an intermediate or end product. Detailed molecular information on changes made using ETGM is provided as part of any EU authorisation of products resulting from the employment of these techniques, and the data are stored in relevant regulatory databases.

- Prior information on the end product or on the exogenous nucleic acid in an end product enables detection with a variety of analytical techniques, which is progressively easier as the size of the exogenous nucleic acid fragment increases.

- Detection is more challenging if no information concerning the introduced changes is available (or cannot be postulated, *e.g.* from

databases of existing authorized GMOs), but a significant attempt can be made through the application of whole genome sequencing (WGS) in combination with bioinformatics, and in such cases detection depends on the availability of a suitable reference (baseline[15]) genome. Nevertheless, it is generally impossible to distinguish the cause of such changes as natural or resulting from the employment of any breeding technique.

Speed and cost to achieve the expected result and degree of maturity for field applications

- The Note makes only qualitative statements about the relative costs and speed of product development. Publicly available qualitative and even quantitative data about development time or costs for different breeding techniques are scarce. Moreover, speed largely depends on the specific trait and on the species into which the genetic alteration is introduced. However, the speed with which mutations can be introduced using NBT is often higher (in particular when using the CRISPR-Cas genome editing system) than that which can be achieved with ETGM and CBT, mainly due to the reduced need for time-consuming screening procedures and/or back-crossing, with correspondingly lower costs. The time and costs related to subsequent regulatory approval are not within the scope of the Note.

- In terms of maturity, the Note makes a qualitative assessment from a purely technical point of view, on how close products of NBT are to field trials and beyond. Detailed publicly available information on such products is however scarce.

Synthetic biology and gene drives

- In synthetic biology, a combination of ETGM and/or NBT techniques is used with computer science and engineering approaches, for example to introduce large sets of genes encoding complete biochemical pathways, or to modify existing or create entirely new, artificial organisms.

[15] The most appropriate reference genome is the one that is obtained from the organism which will be changed, immediately prior to the change being made in order to reduce the incidence of spontaneous mutations to as low a level as possible.

- The NBT can also be used for gene drives which aim at increasing the prevalence of a specific gene in a population to nearly 100%. Further research will be needed in relation to *inter alia* efficiency and safety before organisms to which this approach is applied can be considered for release into the environment.

Guidance for the reader

- The Note is divided into a number of main chapters including an introduction (1), this summary (2), the description of techniques (3), and the comparison of techniques (4). Chapter 3 describes CBT, ETGM, and NBT. CBT and ETGM are described, in addition to the request for a description of NBT in the scoping paper because their description enables the reader's understanding of the NBT and their subsequent comparison, in particular because NBT can be used in combination with CBT and ETGM; and because all techniques are still in use to a greater or lesser degree in parallel.

- Chapter 4 of the Note compares techniques, with the aid of two sets of tables. Both sets of tables compare techniques according to the criteria described in the scoping paper. The first set of tables compares NBT with CBT, and the second, NBT with ETGM. There are some differences between the terms and set of criteria in the scoping paper and those in the tables. These differences reflect the outcome of discussion between experts during the development of the note. Not all techniques described in chapter 3 are compared with one another; only those for which comparisons are relevant and useful (*i.e.* only those techniques which employ similar molecular mechanisms and/or which produce similar end products are compared).

- In addition to the chapters described above, the note also includes acknowledgements, and annexes including the scoping paper, a description of the evidence review methodology, references and a glossary.

Description of techniques

3. DESCRIPTION OF TECHNIQUES

In nature, genetic variety results from mutation and enables populations to adapt to changing environments, driving evolution by natural selection. The different traits which result from genetic variety are expressed as the organism's phenotype. There are several natural processes causing different types of mutation (see Text Box 1 – What causes genetic variety?).

By employing the techniques described in this Note, breeders select and/or introduce desirable traits, affecting the genetic variety of populations of organisms.

Humans have made use of natural variation since first cultivating land and breeding livestock around 13,000 years ago, selecting and retaining organisms suitable for agricultural use (Larson & Fuller, 2014; Stamp & Visser, 2012). In this way, useful traits appearing spontaneously were bred into certain crops or animals by human (rather than natural) selection.

The discovery of the laws of inheritance by Gregor Mendel towards the end of the 19th century accelerated the alteration of the genotypes and phenotypes of plants and animals through human intervention by selective breeding (Borém, Diola, & Fritsche-Neto, 2014; Christou, Savin, Costa-Pierce, Misztal, & Whitelaw, 2013). These CBT rely on genetic variation occurring randomly and are usually restricted by sexual compatibility*, i.e.* they stay within the boundaries of the gene pool of a given population.

Natural genetic variation can be further increased through induced mutagenesis[16], which was first applied around 1920 (Francis, Finer, & Grotewold, 2017). Increasing knowledge of genetics and improved methods of DNA analysis led to an upgrade of conventional breeding techniques to

[16] This Note is intended to describe and compare various techniques from a scientific and technical perspective and not to examine legal issues. Nevertheless, and for clarity, it is noted that according to the relevant European legislation, induced mutagenesis is an established technique of genetic modification, but is exempt from the provisions of this legislation. It is for this reason that induced mutagenesis is considered here a conventional breeding technique.

arrive at marker-assisted selection as of 1978 (Francis et al., 2017; VIB, 2016).

With the invention of recombinant DNA technology in the second half of the twentieth century, it became possible to cut and splice individual DNA molecules together to make entirely new ones. In particular, it has led to the development of transgenesis, in which genomes are altered by the integration of exogenous (*i.e.* from other genomes) DNA fragments (*e.g.* genes). This new technology of genetic modification allows the transfer of genes between even very distantly related organisms. It was developed first in bacteria and the viruses which infect them, and subsequently applied to multi-cellular organisms, including plants and vertebrates. Transgenesis became a powerful tool in research for a better understanding of gene functions and physiological mechanisms, and for breeders by providing access to the full potential of biodiversity. Two major limitations of transgenesis in plants and (non-laboratory) animals should however be noted: (i) that most phenotypic traits are complex, and require more than a single gene and that (ii) it offers no control over where the added genes are inserted into the genome (Nuffield Council on Bioethics, 2016).

Among the NBT described in this Note, the genome editing techniques have attracted the most attention in recent years. Genome editing enables the efficient alteration of genomes in a specific and site-directed way. Instead of random mutation of many genes at the same time (as in CBT) or random insertion of new genes (as in ETGM in plants and animals), genome editing allows the selective mutation of one or a few genes exclusively and the precise modification or replacement of entire genes, whether from closely or distantly related organisms. Other NBT are not intended to alter the genome at all, but rather to temporarily change gene expression patterns in order to adjust the traits of an organism.

Although there has been a succession of many different techniques, they have not replaced each other. Each technique has advantages and disadvantages in specific situations, depending *e.g.* on the respective species, the purpose, the environment and other conditions (VIB, 2016).

Figure 1 gives an overview of historical developments in plant breeding, but the principles are also largely applicable to animals and partially to microorganisms and macroscopic fungi (Francis et al., 2017).

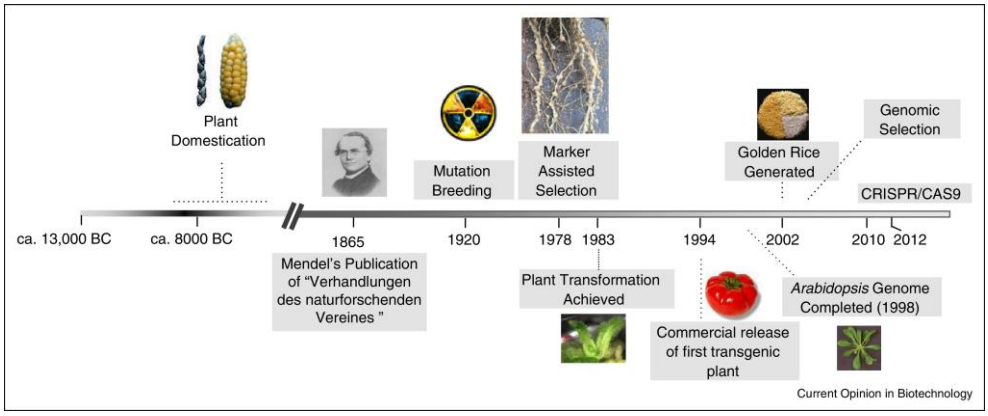

Figure 1 - Timeline of key events in plant breeding

Source: (Francis et al., 2017)

Text Box 1

What causes genetic variety?

Genomes are naturally susceptible to alteration and errors occur every time a cell copies its DNA during cell division (necessary for growth and reproduction). These errors may be neutral, harmful or even lethal, or they may confer a competitive advantage, and are the basis for natural selection. In addition, genomes may be altered by environmental influences, *e.g.* by viral infection and ionising radiation (for example, X-rays and far ultraviolet light), which can disrupt DNA at locations that may be difficult or impossible to predict (Nuffield Council on Bioethics, 2016). Alterations can include smaller changes such as the substitution, insertion or deletion of one nucleotide base pair (collectively known as point mutations) or more gross changes such as insertions or deletions of larger DNA fragments, DNA inversions or translocations (the rearrangements of parts between non-homologous chromosomes). Even the exchange of only one base pair can have a major, sometimes lethal effect, depending on the position where it occurs and whether it can be compensated for (*e.g*. by the other allele).

The consequence of these natural alterations is that genomes at the species level are dynamic, with genes present in every individual (core) and genes in a subset of individuals (dispensable) that collectively constitute the pan-genome. Dispensable

genes may constitute a significant proportion of the pan-genome around 20% in soybean (Li et al., 2014).

Genetic alterations can occur in somatic or germline cells. Somatic cells are those cells that form the body of the organism. In contrast, the germline is a specific tissue developing the (reproductive) haploid germ cells (gametes). If the genetic alteration is present in a gamete which participates in fertilisation, it will be passed on to the next generation.

The following sections describe CBT, ETGM and NBT in plants, animals and microorganisms as well as unintended effects occurring while employing different breeding techniques.

Unintended effects

In principle two different types of unintended effects can occur during breeding: (1) unintended changes and (2) unintended effects of the intended changes. (1) In the first case changes other than the intended ones are introduced into the organism; the type of these unintended changes differs depending on the type of the employed breeding technique and also depending on whether the techniques are employed in plants, animals or micororganisms (this will be described in detail in the respective section below). (2) In the second case the intended changes are successfully introduced into the organism. However, their effect may not be the intended one, which mainly depends on environmental factors (through epigenetic modifications) and the interaction with other genes, which may have an effect on the expression of other genes or the gene itself and thus on the phenotype of the organism. The effects described under (2) are general phenomena which can occur with any breeding technique employed and all unintended effects associated with specific breeding techniques must to be seen in this general context.

The unintended effects described in more detail for the various techniques in the following chapters include so-called 'off-target effects' (a precise change is made to a genetic sequence identical/ similar to that in which the change is intended, but in another location), 'position effects' (the variation of expression exhibited by identical transgenes that insert into different

regions of a genome, often due to regulatory sequences of neighbouring genes) and 'pleiotropic effects' (one gene affects two or more seemingly unrelated phenotypic traits). Such unintended effects of the different breeding techniques are compared in chapter 4.

3.1. Conventional breeding techniques (CBT)

For the purposes of this Note CBT are those techniques, the use of which (with the exception of animal somatic cloning) predates the use of ETGM and the products of which combine traits which pre-exist in the genetic potential of the 'parent' organisms (Acquaah, 2015).

Many CBT are nevertheless informed by the latest developments in biotechnology and molecular biology and by the sequencing and annotation of relevant genomes.

3.1.1. Conventional breeding techniques in plants

There is a wide range of CBT used in plants. For the purposes of this Note, a list based on the techniques established by the European Food Safety Authority (EFSA) is used (EFSA Panel on Genetically Modified Organisms, 2012).

Simple selection

In preferring some plants to others for continued propagation on the basis of their desirable traits (*e.g.* improved palatability or yield), our ancestors practiced simple selection. These are among the traits displayed in the plant's phenotype, which results from the expression of the plant's genotype, in interaction with the environment. Selecting for a particular phenotype has a corresponding and progressive effect on the plant's genotype: plants (or their seeds) displaying the preferred phenotype are preferentially saved and replanted, shifting the genetic population so that it is dominated by the preferred genotype. The resulting product (variety or population) usually maintains some heterogeneity relative to the global population, but may also be an inbred line, with much reduced heterogeneity (Centre for Biosafety and Sustainability, 2016) All widely used modern crops are the product of (at least) simple selection (Borrelli et al., 2014; Sakuma, Salomon, & Komatsuda, 2011).

Sexual crosses

Simple selection for desirable traits is accelerated by the employment of sexual crossing, the most frequently used plant breeding technique (Van De

Wiel et al., 2010; Xia, 2009). It is based on the Mendelian laws of inheritance. Figure 2 illustrates these laws, and introduces the concept of alleles, which are variant forms of a given gene which may correspond to different phenotypes. The aim is to bring together desired traits found in different plant varieties via cross-pollination.

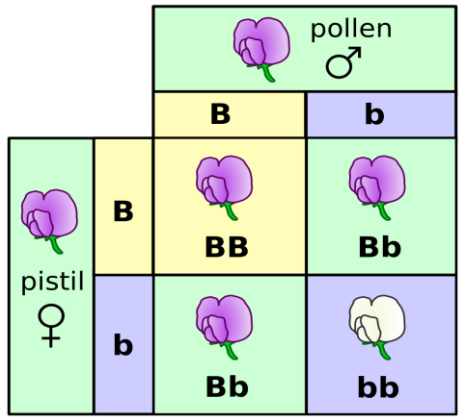

Figure 2 - Mendelian laws of inheritance

Source: Wikipedia/commons

Intraspecies and interspecies crossing

The procedure involves crossing plant varieties which have already been selected for distinct desired traits with each other, or with wild relatives of plants expressing desired traits, and the subsequent selection of progeny. Backcrossing involves repeated crossing of an interesting genotype with the same selected parent for introducing an interesting character in an elite genotypic background. Reduction of vigour and size (inbreeding) can be observed when allogamous plants are backcrossed but not with autogamous lines which correspond to pure lineages (Khan, 2015).

The subsequent selection of interesting plants with the desired combined traits has previously been based upon the identification of phenotypic characteristics such as colour and yield. One of the tools which is now extensively used and which make it easier and faster to select plant traits is marker-assisted selection (MAS), which is based on the molecular detection of genomic markers closely associated to the specific trait (Collard & Mackill, 2008; Kadirvel, Senthilvel, Geethanjali, Sujatha, & Varaprasad,

2015; Zhang, Fang, Zhou, Sanogo, & Ma, 2014). Recently, in some cases, automated phenotyping platforms have also been used for this purpose (Christou et al., 2013; Jin & Nassirou, 2015; Tai, 2017).

In many cases crossing cannot be accomplished due to sexual incompatibility, which limits the possibilities of introducing desired traits into crop plants (Centre for Biosafety and Sustainability, 2016) and alternative procedures may be used such as bridge crosses, embryo rescue (Bharadwaj, 2016; Lusser, Parisi, Plan, & Rodríguez-Cerezo, 2011) or somatic hybridisation.

Bridge crosses

When a direct cross between two species, A and B, is not possible, an intermediate crossing with a third species, C, which is compatible with both species, may bridge the crossing barrier. First, a cross is made between A and C, and the resulting interspecific hybrid is subsequently crossed with species B. So, by indirectly crossing, the genomes or segments thereof from species A and B can be combined (Van De Wiel et al., 2010).

Embryo rescue

Sometimes technical intervention is required to facilitate the generation of crossing products between species, which are otherwise unable to produce viable hybrids. Some plants will cross-pollinate and the resulting fertilized hybrid embryo develops but is unable to mature and sprout. This problem can be worked around by pollinating naturally, then removing the plant embryo before it stops growing and placing it in a tissue-culture environment where it can complete its development. One of the primary uses of embryo rescue has been to produce interspecific and even intergeneric hybrids (Acquaah, 2013).

Hybridization for vigour (heterosis)

A specific use of sexual crossing is based on the exploitation of heterosis (the phenotypic superiority of a cross over its parents).

When highly inbred varieties are crossed with other inbred varieties, very vigorous, large sized, large-fruited plants may result from the effect known

as heterosis (yield advantages range between 5% and 50%, depending on the crop). This approach has been successfully applied first to maize and then to other species such as sunflower, sorghum, rapeseed, and its use in wheat has been investigated (Lippman & Zamir, 2007). Today nearly all maize is hybrid, although the farmers must buy new hybrid seed every year, because the heterosis effect is lost in progeny.

Somatic hybridisation

Somatic hybridisation relies on tissue culture to combine genes from plant varieties which are sexually incompatible. In this technique, somatic cells from two varieties of plants are stripped of their protective cell walls and the resulting protoplasts are pooled. Various methods such as poly-ethylene-glycol (PEG) or electrical shock are employed to fuse these protoplasts.

When two protoplasts fuse, the resulting somatic hybrid contains the genetic material from both plant sources. This includes (i) two sets of parental chromosomes, which may differ in number, if the parents are taxonomically distinct and (ii) various organelles. If compatible, and if parental nuclei fuse, a new hybrid plant may develop that carries the chromosomes of both parents. Very often, mitochondrial parental genomes recombine giving rise to new genomes. By a process of sorting out, cytoplasmic organelle populations are obtained in one or the other nuclear context (no nuclear fusion). The resulting plants are called cybrids (for cytoplasmic hybrids).

The presence of two sets of parental chromosomes leads to polyploidy (see below, 'doubled haploids and polyploidy induction').

Somatic hybridisation is however not common in plant breeding, as its successful applications to create new chromosome associations have not extended much beyond what is possible using other CBT (Committee on Identifying and Assessing Unintended Effects of Genetically Engineered Foods on Human Health, 2004). Cybrids are largely used worldwide in *Brassica* crops.

Mutation breeding

Spontaneous DNA mutations naturally occur (see Text Box 1) and are usually either neutral or harmful. More rarely, a mutation will result in the expression of a novel and desirable trait.

Plant breeders attempt to increase and accelerate these events by inducing mutations (Suprasanna, Mirajkar, & Bhagwat, 2015) and selecting for rare desirable traits. Mutation breeding involves exposing plants or seeds to physical (radiation *e.g.* X-rays) or chemical (*e.g.* ethyl methane-sulfonate (EMS) mutagenic agents), which induce random changes in DNA sequences throughout the genome. The mutation can consist of changes in one nucleotide position only, or sometimes (more frequently after radiation with X-rays) of more complex changes such as major rearrangements in the DNA (inversion, translocation) or the elimination of DNA fragments (deletion).

Because the mutations are random, very large cohorts of mutant plants are required to identify the rare, useful mutations, and once identified, backcrossing is employed to eliminate unwanted traits. Nevertheless, crops derived from mutation breeding are still likely to carry DNA alterations beyond the specific mutation that provided the desired trait. To date, more than 3,200 different commercially available crop varieties have been developed worldwide using induced mutagenesis (Jankowicz-Cieslak, Tai, Kumlehn, & Till Editors, 2017). Examples of applications are a range of new rice and banana varieties, which are cultivated and consumed in great quantities. The pink grapefruit is another well-known product (VIB, 2013).

Translocation breeding

In case a chromosome with a desired gene carries undesirable other genes, they can be separated by inducing (*e.g.* by irradiation or by bridge crossing) translocations of parts of the donor species chromosome onto the recipient species chromosome(s) (Sears, E.R. (1956) in (Dhillon, 2011)). The transfer of leaf-rust resistance from *Aegilops umbellulata* (wild grass) to *Triticum aestivum* (common, or bread wheat) is an example of the use of

an intermediate bridge cross in this technique (Hartung & Schiemann, 2014).

Doubled haploids and polyploidy induction

Most plant species are diploid, meaning that they have two sets of corresponding (homologous) chromosomes in their cell nuclei. Homologous chromosomes may have allelic variants of the same genes. In sexual reproduction, haploid gametes are formed which contain one set of chromosomes and the subsequent combination of gametes (and thus of sets of alleles) determines the genotype (and the phenotype) of the offspring.

Viable haploid plants can originate spontaneously in nature or can be induced, including through the tissue culture of gametes. The subsequent application of the chemical colchicine to haploid plants prevents mitotic cell division and results in so-called 'doubled haploid' (DH) plants (Acquaah, 2015). These plants are homozygotic rather than heterozygotic in that they do not display allelic variation and can be used to produce genetically identical progeny or in sexual crosses to obtain offspring with hybrid vigour (heterosis).

These techniques are used by breeders to avoid time consuming and costly backcrossing. Using DH production systems, homozygosity is achieved in one generation, eliminating the need for several generations of self-pollination. The time saving is substantial, particularly in biennial crops and in crops with a long juvenile period. In some cases haploidy may be the only way to develop inbred lines (Singh & Singh, 2015).

Plants with three or more chromosome sets are known as polyploid (Soltis & Soltis, 2012). A number of well-known crops are polyploid, for instance wheat (which is allopolyploid, meaning that it has 3 complete sets of chromosomes from 3 different species). The main effects of polyploidy are often increased size, robustness and genetic variability, but may also include reduced fertility and growth rate (Centre for Biosafety and Sustainability, 2016).

Text Box 2

Somaclonal variation

A phenomenon which is relevant for all plant breeding techniques which make use of *in vitro* cultures of plant cells or tissues (callus), is the appearance of spontaneous genetic and epigenetic changes called somaclonal variations, in particular after multiple cell passages in culture (Krishna et al., 2016). In *Arabidopsis thaliana* plants (a small flowering plant considered a weed, used as a model organism in molecular biology) regenerated from callus, about 100 times more mutations were present than in plants propagated from seed (C. Jiang et al., 2011). Similar results have been obtained in rice, where the mutation rate in plants obtained through *in vitro* culture was about 250 fold increased (Miyao et al., 2012). This phenomenon has sometimes been exploited in conventional breeding for creating genetic variability.

Unintended effects of Conventional Breeding Techniques in plants

When sexual crossing is employed to introduce a desired trait from a donor plant into a recipient plant, two haploid parental genomes are brought together in offspring. First generation offspring inheriting the desired trait from one parent will also inherit additional unwanted traits from both parents (since offspring inherit ~50% of each parent's complement of DNA). These alleles can have unintended effects (including pleiotropic effects and effects on the expression of other genes).

The phenomenon of meiotic recombination (in which homologous chromosomes 'cross over' and enable the rearrangement of alleles during the formation of gametes) means that further breeding may result in subsequent generations displaying a proportionally greater amount of unwanted traits. This can be controlled with backcrossing to isolate the desired trait from undesired traits. However, it is difficult to eliminate all undesired traits with backcrossing, in particular those traits of which the encoding genes are located closely to the gene(s) encoding the desired trait in the genome.

The spontaneous mutation rate is about 7×10^{-9} base substitutions, per site, per generation. This results in one base substitution per generation in a

genome the size of *Arabidopsis thaliana* (Ossowski et al., 2010). This means that unintended effects can also accumulate in sexual crossing.

Induced mutagenesis, depending on intensity and concentration of the mutagenic agent, can increase this mutation rate by a factor of approximately 500 (Cooper et al., 2008; Jander et al., 2003; Till et al., 2007).

All mutations occurring in addition to the mutations conferring the desired trait can be considered 'off-target'. There is a high probability that the random mutations in some genes will also influence the expression of other genes. These random mutations can only be detected through WGS with the restriction that WGS may not detect all mutations if the obtained genome sequence is incomplete, *e.g.* due to the structural features of the genome. Typically, however, the selected plants with the desired traits will still contain a high number of undetected mutations, in particular if they do not cause disadvantageous phenotypic traits (Acquaah, 2015; Popova, Mukund, Kim, & Saxena, 2015).

3.1.2. Conventional breeding techniques in farm animals

From natural mating to modern breeding programmes

The breeding of domestic animals has taken place for thousands of years while people have kept animals in their proximity and used their products (Larson & Fuller, 2014). Using the technical options that were available in each time period, humans have propagated those populations deemed useful for their needs. Simple selection mostly occurred according to the phenotypic traits and a vast number of phenotypically different breeds with desirable traits have resulted from the continuous breeding efforts in an evolutionarily short time period. A good example are cattle, for which nowadays >800 breeds can be found worldwide (Orozco-terWengel et al., 2015).

Modern, scientifically based animal breeding strategies have existed for ~60 years, and are mainly based on several biotechnological procedures, of which artificial insemination (AI) is the most prominent one. In the second half of

the 1980s, embryo transfer (ET) technology has been introduced into animal breeding and allowed for the first time a better exploitation of the genetic potential of female animals. Additional embryo related bio-techniques, such as *in vitro* production of embryos and sexing were developed and rapidly integrated into existing breeding programs.

These breeding strategies predominantly based on population genetics, as well as AI and ET-technologies led to significant increases in the performance of domestic animals with regard to the provision of abundant amounts of valuable animal proteins, including meat, milk and eggs.

Reproductive biotechnologies have recently been complemented by new developments in molecular genetics that are compatible to further increase the selection of valuable breeding animals. The genomes of the major agricultural animals have been sequenced and annotated, including cattle, pigs, chicken, sheep, poultry, and bee ("Ensembl genome browser," 2017). This has allowed the development of improved breeding strategies initially based on marker assisted selection (MAS) and other more sophisticated techniques.

Artificial insemination (AI) from selected sires

AI allows the effective propagation of the genetic potential of valuable sires (male parent of an animal) within a population (Hasler, 2014) and is employed in more than 90% of all sexually mature female dairy cattle in countries with advanced breeding programs. Application of AI is also steadily increasing in pigs, where now on a global scale >50% of sexually matured sows are fertilized by AI (Kues, Rath, & Niemann, 2008). AI is less frequently used in small ruminants.

Embryo transfer (ET) technology

ET allows the exploitation of the genetic potential of female animals, albeit at limited efficiency since the pool of female gametes (oocytes, or eggs) is already determined at birth and only a small fraction can develop to mature oocytes. Approximately 660,000 *in vivo* collected bovine embryos have been transferred worldwide in 2015, of which ~50% were used after

freezing and thawing; in addition, a significant number of the transferred embryos (~550,000) were derived from *in vitro* production (*in vitro* maturation, fertilization and culture of embryos) (Perry, 2016). Embryo collection and transfers can be non-surgically performed in cattle, thus facilitating application under field conditions. ET-technology requires surgical or endoscopic techniques in pigs and small ruminants, thereby limiting application to very specific purposes. In contrast to AI, embryo transfer is predominantly used in the most valuable (top 1%) females of a given breeding population.

Oocyte collection from selected dams and *in vitro* production of embryos

Ultrasound guided follicular aspiration or Ovum Pick Up (OPU) has emerged as an alternative to conventional bovine embryo recovery programs based on superovulation of the donor animals. OPU can be employed regardless of the reproductive status of the donor. The high number of viable oocytes, which can be collected from a single animal within a relatively short period of time, is an important advantage of this technology. On a yearly basis, OPU is 3.5 to 5 times more efficient for embryo production than superovulation followed by uterine flushing of embryos (Oropeza, Hadeler, & Niemann, 2006). Besides cattle, OPU is also applied in the horse.

Collected oocytes are usually subjected to an *in vitro* embryo production (IVP) system, including *in vitro* maturation (IVM) to obtain fertilizable oocytes; followed by *in vitro* fertilization (IVF), and ultimately concluded by *in vitro* culture (IVC). Current IVP protocols are compatible with similar pregnancy rates as after transfer of *in vivo* produced embryos. IVP has many application fields, including multiplying embryos from valuable females at nearly any stage of the reproductive status, inexpensive production of calves for example in beef production systems, or collection of oocytes from pre-pubertal donors. Thus, OPU followed by an efficient IVP system has emerged as a powerful tool in modern cattle breeding to achieve higher rates of genetic improvement (Kropp, Peñagaricano, Salih, & Khatib, 2014; Seidel, 2016). OPU and IVP are widely applied around the globe, in particular in South America.

Long term storage of gametes and embryos

Cryopreservation allows the long-term preservation of gametes and embryos at ultra-low temperatures in a state of suspended animation. Cryopreservation protocols essentially include two methods: conventional, slow freezing and vitrification (rapid freezing), both followed by plunging into liquid nitrogen. Freezing of germplasm involves the preservation of oocytes, sperm and embryos of a wide variety of domestic animal species, including cattle, sheep and goats, horse, and to some extent also pigs and is widely used in breeding programs. If correctly applied, current freezing protocols are compatible with virtually no losses after thawing and thus allow distribution of animal genetic resources around the globe. Moreover, the establishment of germplasm banks has significantly contributed to protecting rare and endangered species from extinction. One of the biggest concerns regarding cryopreservation pertains to contamination. The risk of pathogen infection of oocytes, sperm and embryos through in vitro fertilization (IVF), artificial insemination (AI) and/or embryo transfer (ET) is a matter of concern for health officials and requires strict hygienic measures to prevent spread of pathogens (Bielanski, 2012; Mandawala, Harvey, Roy, & Fowler, 2016; Saragusty & Arav, 2011).

Sex determination (Sperm sexing, Embryo genotyping)

Sex in mammalian reproduction is determined by the chromosomal set-up of the sperm that can either carry an X- or Y-chromosome that fertilizes the oocyte carrying one X-chromosome, resulting in an XY (male) or XX (female) configuration in the developing embryo. Spermatozoa from livestock species show a difference in the relative amount of DNA between X- and Y-chromosome bearing sperm in the range of 3% to 5%. This difference in DNA contents between X- and Y-bearing sperm can be exploited to effectively separate populations of X- and Y-sperm in mammals. It is increasingly being used in livestock production; recent figures indicate that 6-10% of embryos are transferred after fertilization with sex sorted semen (Perry, 2016). The reliable control of the sex ratio permits faster genetic progress, higher productivity, improves animal

welfare by reducing the incidence of difficult births in cattle, avoiding castration of male pigs, and producing less environmental impact due to elimination of animals with the unwanted sex. Current sexing technology is commercially available for cattle breeding and could evolve as a major tool towards the implementation of more efficient breeding and production schemes. More research is needed to allow commercial application of sex sorted semen for other livestock species (Rath et al., 2013).

The sex can also be reliably determined in small biopsies of 10-12 cells from preimplantation embryos, preferably at the blastocyst stage. In case of male embryos, a Y-chromosome specific stretch of DNA is amplified which allows unequivocal discrimination between male and female embryos. Embryo sexing can be done within a few hours prior to transfer, thus allowing the production of calves of predetermined sex. Using the same approach, it is now also possible to genotype embryos in the context of MAS and other such techniques (Humblot et al., 2010).

Emerging technology: Somatic cloning (somatic cell nuclear transfer, SCNT)

Somatic cloning (also known as somatic cell nuclear transfer or SCNT) is a laboratory technique for creating new organisms that are largely genetically identical to existing organisms. Cloning in mammals involves replacing the genetic material of an egg with the genetic material of a somatic cell from an embryo or adult. The egg then develops into a full organism genetically identical to the donor organism. Protocols for somatic cloning of livestock species have been significantly improved over the past years, thus allowing field applications. One of the remaining drawbacks of SCNT is an elevated proportion of embryonic mortality (EFSA Panel on Genetically Modified Organisms, 2012b), while the incidence of oversized offspring, initially observed in ruminants after cloning, does not seem to be a major problem anymore (Han et al., 2015).

The birth of "Dolly" in 1996, the first mammal cloned from an adult donor cell sparked a flurry of research activities to improve cloning technology and to understand the underlying mechanism of epigenetic reprogramming of

the transferred somatic cell nucleus by yet unknown factors in the recipient ooplasm. Epigenetic reprogramming entails modifications at the level of DNA histone proteins, and is associated with significant changes in the expression profile of the transferred somatic donor cell leading to the formation of a pluripotent cell, while leaving the DNA structure intact. The most critical factor is epigenetic reprogramming of the transferred somatic cell nucleus from its differentiated status into the totipotent state of the early embryo. This involves erasure of the gene expression program of the respective donor cell and the establishment of the well-orchestrated sequence of expression of an estimated number of 10,000–12,000 genes regulating embryonic and foetal development (Niemann, Tian, King, & Lee, 2008).

SCNT has been successful in at least 24 mammalian species. Cloned offspring (*e.g.* cattle, pigs) are not different from age matched controls and appear healthy and develop normally (Niemann, Kues, Lucas-Hahn, & Carnwath, 2011).

SCNT is already applied for multiplication of valuable breeding animals, for the maintenance of genetic resources, to reproduce high performing racehorses.

Unintended effects of CBT in animals

The mutation rate in preimplantation embryos of farm animals is relatively low with one mutation occurring in 10^{11} base pairs per cell cycle. Detailed studies for the frequency of postnatal mutations in farm animals are lacking. Pleiotropic effects occur with low frequency in CBT and can be used in the breeding strategy (W. Kues & Niemann, 2011).

3.1.3. Conventional breeding techniques for microbial strain development and improvement

Humans have used microbes for centuries to produce food. Wine, bread, and cheese are common examples of foods that depend on microbial ingredients and activities. Today, microorganisms play even more significant roles in food production. They serve primary and secondary roles in food fermentation and in preventing food spoilage, and they can produce

enzymes or other metabolites used in food production and processing. The most widely used organisms are lactic acid bacteria (LAB) and yeasts (in particular *Saccharomyces cerevisiae*) (Acquaah, 2015).

Microorganisms relevant for food and feed production are also those which form complex microbial communities (microbiomes) tightly associated with animal and plant organisms (hosts). For instance, the microbiomes of both the animal gut and of plants are known for their importance for the host's nutrient uptake, protection against biotic (pathogens) and abiotic stress, as well as for providing metabolic capabilities (Ramírez-Puebla et al., 2013). The lactic acid bacteria (LAB) group is nowadays used as probiotics, microbes that are thought to provide health benefits when consumed.

Bacteria reproduce by binary fission in which a single bacterial cell divides into two identical daughter cells (clones).They have a genome consisting of one circular DNA molecule, but may have additional smaller circular DNA molecules known as a plasmids which encodes additional functions. Variety arising during binary fission is due to errors in the copying of DNA.

Yeasts are single-cell fungi, which are eukaryotes with a cell nucleus containing most of the DNA and mitochondria with the remaining small part. The nuclear genome of yeasts consists of several linear chromosomes and the mitochondria have a small circular genome. Like other eukaryotes yeasts reproduce by mitosis, a process in which the duplicated chromosomes in the nucleus are divided over the two nuclei in the daughter cells and the daughter also receive a complement of the mitochondria. This produces genetically identical daughter cells (clones). Yeasts can exist in the haploid and diploid state. Haploids of opposite "mating type" can fuse to form a diploid, which can undergo meiosis to form haploid spores. This sexual reproduction through meiosis leads to variety within populations.

Genetic variety within bacterial populations arises from a number of different natural processes, which enable the exchange of DNA, or 'horizontal gene transfer' between bacteria.

Conjugation is a natural process of genetic exchange whereby DNA is transferred from one bacterial cell to another, which requires cell-to-cell

contact. Conjugation can occur not only between strains but also between different bacterial genera (Steenson & Klaenhammer, 1987). DNA can be also transferred by conjugation from bacterial prokaryotic cells into eukaryotic host cells *in vitro* culture, including yeast, plants and mammalian cells (Llosa, Gomis-Rüth, Coll, & De la Cruz, 2002). *In vitro Agrobacterium* can deliver T-DNA not only into plant cells, but also into yeast and fungal cells (Bundock, den Dulk-Ras, Beijersbergen, & Hooykaas, 1995).

Transduction is the process by which foreign DNA is introduced into a cell by a virus, and this DNA incorporated into the host's genetic information (Hartl, 1998). Transduction is one example of a horizontal gene transfer mechanism which is responsible for the transfer of pieces of DNA between closely related bacteria.

Another example of horizontal gene transfer is natural genetic transformation which is the active uptake of extracellular DNA (of any origin) by microbial cells and the heritable incorporation of its genetic information (Nielsen, Johnsen, Bensasson, & Daffonchio, 2007). Natural transformation has been detected among bacteria (and other microorganisms including *archaea*) from all trophic and taxonomic groups including industrially important bacteria such as *Bacillus* species used for enzyme production (Nijland, Burgess, Errington, & Veening, 2010) and lactic acid bacteria with food applications (Helmark, Hansen, Jelle, Sørensen, & Jensen, 2004).

Not all bacteria are naturally capable of taking up exogenous DNA (see also sections 3.2 and 3.2.3). However, this ability can be induced via stress (*e.g.* thermal or electric shock), thereby increasing the cell membrane's permeability to DNA. Up-taken DNA may be propagated to the daughter cells either if it can act as an autonomously replicating element (*e.g*. a plasmid) or after integration within the host genome.

Self-cloning is defined as the re-introduction of a host's own DNA which has been altered, or the introduction of DNA from a closely related strain of the same species[17].

Mutagenesis and selection

The earliest and most widely used technique to generate and select microorganisms with preferred characteristics is induced mutation using physical or chemical agents, followed by an enrichment or selection process of mutants with preferred characteristics. Recent examples of applications to lactic acid bacteria used for food production are reviewed in (Derkx et al., 2014).

Protoplast fusion

A less common, but still useful (Adrio & Demain, 2006), method has been to use protoplast fusion[18] to facilitate recombination between two microbial strains with desired but unique characteristics. Protoplast fusion was classically used as a mapping method for determining genetic linkage in bacteria and more recently has been used successfully to produce strains with desired characteristics (Patnaik et al., 2002).

Unintended effects of CBT in microorganisms

The spontaneous mutation rate in *Escherichia coli* with a genome size of $5x10^6$ base pairs (bp) is about 10^{-10} mutations per bp per replication, corresponding to about one mutation every 1000 generations (Blattner et al., 1997). Both spontaneous and induced mutations can influence the expression of other genes and have pleiotropic effects. The probability depends on the mutation rate (Committee on Identifying and Assessing Unintended Effects of Genetically Engineered Foods on Human Health,

[17] This Note is intended to describe and compare various techniques from a scientific and technical perspective and not to examine legal issues. Nevertheless, and for clarity, it is noted that according to the relevant European legislation, self-cloning of non-pathogenic microorganisms is not considered to lead to a GMO as long as containment of the organism is guaranteed.

[18] This Note is intended to describe and compare various techniques from a scientific and technical perspective and not to examine legal issues. Nevertheless, and for clarity, it is noted that according to the relevant legislation, protoplast fusion is excluded from that legislation under certain circumstances and is for the purposes of this Note treated as a conventional breeding technique.

2004). Natural isolates of microorganisms are known to acquire genetic changes upon laboratory domestication (Liu et al, 2017).

3.2. Established techniques of genetic modification (ETGM) in biotechnology

The development of ETGM in biotechnology towards the end of the 1970s and the beginning of the 1980s enabled the insertion of genetic information into an organism regardless of sexual compatibility, including the inter-kingdom transfer of genes. Thus the gene pool available for improving an organism can be expanded well beyond sexually compatible species (Committee on Identifying and Assessing Unintended Effects of Genetically Engineered Foods on Human Health, 2004).

Exogenous DNA entering a recipient cell will only be stably maintained and expressed either if it can integrate into the host genome or if it can replicate independently (as part of a plasmid). Bacteria and yeast integrate exogenous DNA into the host genome by the process of homologous recombination (HR see 3.3.1), when the exogenous DNA shares a stretch of sequence identity with the host genome. If such homologous stretches are absent, integration only occurs very rarely and fairly randomly. This is very different in plant and animal cells, where any DNA segment can integrate into the genome with similar efficiency by a process of non-homologous end joining (NHEJ, see 3.3.1). If a stretch of homology with the host genome is present on the exogenous DNA, integration may also occur by HR in plant and animal cells, but this is a rare event (frequency 1,000-100,000 times lower depending on the gene and size of homology) (Kumar, Barone, & Smith, 2016; Steinert, Schiml, & Puchta, 2016).

In transient expression, which is preferred for some applications, the introduced nucleic acid sequence is not stably replicated and will be diluted through mitosis during cell division or degraded. In this case the cells express the introduced gene for a limited period of time, usually several days (Liu, Yuan, & Stewart Jr., 2013b).

The most common ETGM used across plants, animals and microorganisms employs recombinant nucleic acids, the use of which was pioneered in bacteria by Boyer and Cohen (Borém et al., 2014). A nucleic acid (usually DNA) sequence corresponding to a desired trait is combined with other

genetic elements, which enable *e.g.* its expression, in the recipient organism.

Typically, the DNA to be inserted contains one or several exogenous gene(s), controlling one or several trait(s) of interest, combined with a vector providing a promoter, a terminator, an origin of replication, a selectable marker gene and a multiple cloning site (Khan et al., 2016). This set of molecular components is usually called a "construct". The promoter controls the transcription of the DNA into RNA (Figure 3), while the terminator ends the transcription.

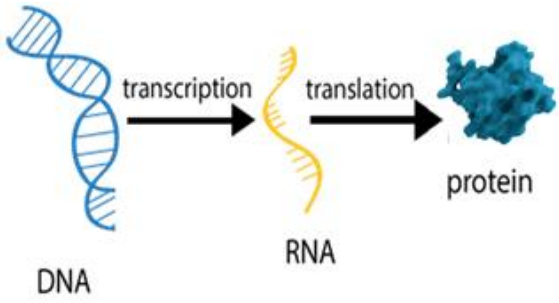

Figure 3 - Gene expression

Source: Authors'

The origin of replication is necessary to initiate the autonomous replication of the introduced DNA in the recipient microbial cell, and the multiple cloning site enables the precise integration of DNA sequences. The selectable marker provides a means for identifying cells which have been transformed with the construct bearing the new gene (Figure 4). In plant and animal cells stable maintenance is only possible by integration in the host genome. Alternatively, genes can be linked to a viral vector which can replicate and spread through the organism, but not to the next generation.

Selectable markers might encode fluorescence (enabling visual discrimination of transformed and non-transformed cells) or antibiotic resistance (enabling discrimination with the use of antibiotics). Other tests can be conducted to confirm that an organism contains the foreign DNA segment, such as Polymerase Chain Reaction (PCR), Southern hybridization, and DNA sequencing. These tests can also confirm the

chromosomal location (where the gene has been inserted) and copy number (how many times it has been inserted) of the inserted DNA.

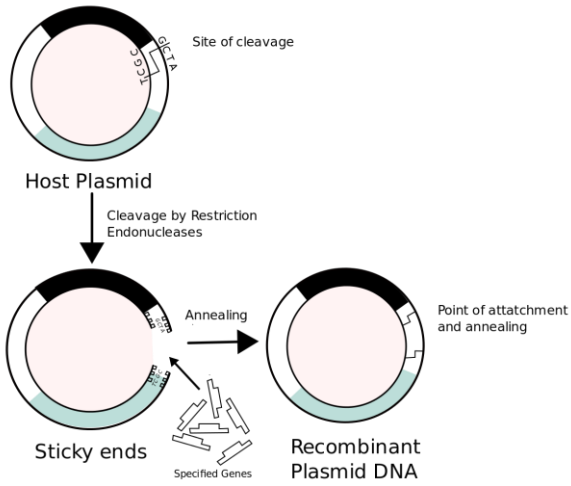

Figure 4 - Recombinant formation of plasmids

Source: Wikipedia

A variety of techniques exists for the introduction of heritable material into cells with some specificities according to use in plants, animals or microorganisms. These techniques include, but are not limited to, incubation with divalent cations (Ca^{2+}) in combination with heat shock, electroporation, enzymatic treatment, *Agrobacterium*-mediated transformation, particle bombardment, viral infection, micro- and macro-injection, microencapsulation, cell fusion and hybridisation. These techniques are explained in the relevant sections.

Depending on the type of vector and host cell used, the process of the genetic modification of a cell resulting from the direct uptake and incorporation of exogenous DNA from its surroundings through the cell membrane is called transformation or transfection. Transformation usually refers to non-viral DNA transfer (with chromosomal DNA or plasmids) in bacteria as well as non-animal eukaryotic cells (plants and yeast). Transfection is the process of introducing naked or purified nucleic acids by non-viral methods into animal cells. Transduction is used in bacteria to describe bacteriophage-mediated gene transfer and is also often used to describe virus-mediated gene transfer into eukaryotic cells.

3.2.1. Plants

Several methods are available for genetic transformation of plant cells, which either require the removal of the cell wall (protoplast transformation) (Pacher & Puchta, 2016; Royal Society, 2016) or allow the transfer of nucleic acids into cells with an intact cell wall or intact tissues or even complete plants. The most common methods include the transformation by a gene vector of the soil bacterium *Agrobacterium tumefaciens* (Figure 5) or the use of mechanical methods such as particle acceleration; the choice among these depends on the target plant species as well as on the intended product and application.

Agrobacterium tumefaciens has the natural ability to transfer DNA to plant cells. This bacterium naturally infects many host plants and subsequently builds a part of its own genetic material into the plant's DNA. The section of the bacterial DNA that is usually inserted by *Agrobacterium* into the plant cell DNA can be replaced with a gene controlling a trait which is useful for agriculture. The first genetically modified plants, tobacco plants engineered to be resistant to herbicides, were developed in the early 1980s using this method.

The particle acceleration method, also sometimes called particle bombardment, the gene gun or 'biolistics', involves coating microscopic particles of *e.g.* gold with the DNA that is to be inserted into the plant. These gold particles are then "shot" under high pressure into the plant tissue. In some cases, the DNA penetrates the nucleus where it is sometimes spontaneously incorporated into the plant's DNA. In comparison with *Agrobacterium*, this method leaves more to chance and is less efficient. However, this remains the most successful way to genetically modify plants that are difficult to infect with *Agrobacterium*.

Plants may also be transformed by viral vectors, either by direct infection or through mediation of *Agrobacterium* (agro-infection). In agro-infection the virus is cloned between the border repeats of a T-DNA vector that is transferred by Agrobacterium into plant cells. In the transformed plant cells the virus "escapes" from the T-DNA, replicates and spreads through the

plant. This may employ either an RNA virus such as Tobacco Mosaic Virus (TMV) or a DNA virus such as a geminivirus. As the virus may be present in high copy number and systemically spread through the entire plant, viral vectors are preferentially used for the large-scale production of proteins of industrial importance, such as antibodies and vaccine antigens, as the end product can be purified of any viral remnants.

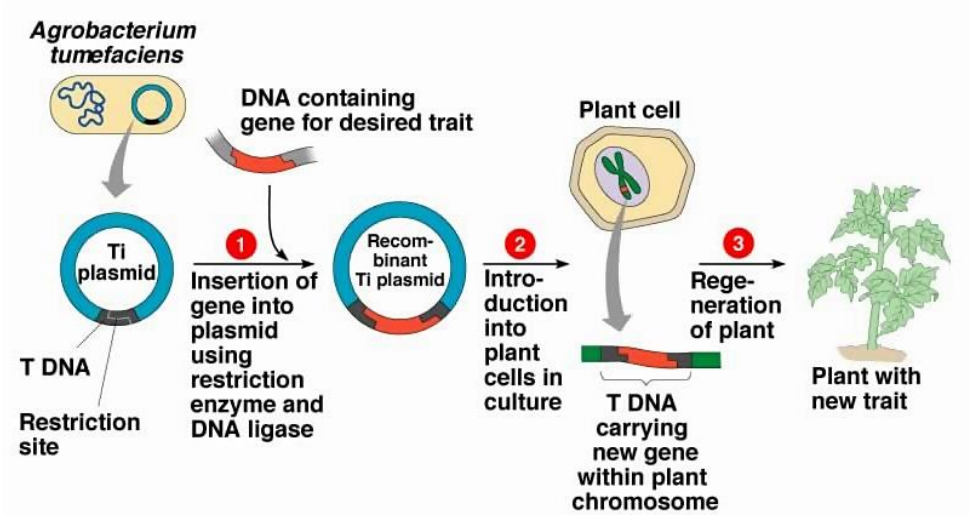

Figure 5 - Transformation with Agrobacterium tumefaciens

Source: Creative Commons

Stable transformation relies on the integration of the introduced DNA segment in one of the chromosomes of the plant cell. Integration occurs at a fairly random position in the genome and expression of the *transgenes* may depend on the exact position (position effect) as well as the copy number of the integrated segment. Single copy insertions are usually preferred as these generally suffer the least from gene silencing effects in the host.

Plants can be regenerated from single cells or protoplasts. Efficiency may depend on the tissue from which the cells were obtained and also on the species or even the cultivar used. If such single plant cells are transformed with exogenous DNA and then regenerated into complete fertile plants, transgenic plants are obtained.

Most of the commercially successful plants produced with ETGM have been transgenic field crops, first commercialised in 1996, which account for 1.0 billion hectares of soybean, 0.6 billion hectares of maize, 0.3 billion hectares of cotton and 0.1 billion hectares of canola worldwide, equalling 83% of the soybean, 29% of the corn, 75% of the cotton, and 24% of the canola grown worldwide (Clive, 2015). These transgenic plants display new characteristics for herbicide and insect resistance with the majority of new genetic components stemming from bacteria, viruses and other plants.

Other examples of transgenic crops, vegetables and fruit include virus resistant squash, Bt potato (a potato transformed with a gene encoding a protein of *Bacillus thuringiensis* conferring resistance against the potato beetle), low polyacrylamide (when cooked at high temperature) potato, virus resistant papaya, herbicide-tolerant resistant flax, and non-browning apple (Francis et al., 2017; VIB, 2016).

Unintended effects of ETGM in plants

One or several copies of the transgene or fragments thereof are inserted fairly randomly at one or several positions in the plant genome. The expression pattern of the transgene depends on its exact position (position effects) and copy number. In fact, since each transgenic organism has the transgene in a different location each transgenic organism has the potential for a unique expression pattern.

This random location of the inserted gene may induce insertional mutagenesis and/or influence the expression of other genes, depending on its exact position. The position and copy number of the inserted DNA can be detected through Southern blots and PCR, if the sequence of the vector and the inserted gene are known (which is usually the case). WGS allows the detection of unknown inserted DNA sequences with the restriction that some insertions (in particular short fragments) may be missed, if the WGS is incomplete, *e.g.* due to the structural features of the genome. It is important to note that changes detected in WGS may not necessarily result from the genetic modification but may be a result of spontaneous mutations including those arising from somaclonal variation (see text box 2) effects

(especially after protoplast transformation). If, however, the changes are not present in a control genome which was sequenced immediately prior to the genetic transformation (the reference genome) there is an increased likelihood that the detected changes are the result of the genetic modification. Regenerated plants must be screened for phenotypic characteristics in order to eliminate those with unwanted traits. In addition, only those plants will be selected as end product which contain only one copy of the introduced gene.

3.2.2. Animals

The first transgenic livestock were reported in 1985 (Hammer et al., 1985). These were produced by microinjection of foreign DNA into zygotic pronuclei. Microinjection is the use of a glass micropipette to inject a substance at a microscopic or borderline macroscopic level, and it had originally been used for genetic modification of mouse embryos (Gordon, Scangos, Plotkin, Barbosa, & Ruddle, 1980).

Microinjection was the method of choice for more than 20 years, but it is increasingly being replaced by more efficient protocols based on somatic cell nuclear transfer (SCNT) which are also compatible with targeted genetic modifications. Microinjection has several major shortcomings, including low efficiency, random integration and variable expression patterns which mainly reflect the site of integration (so-called position effect). Both techniques are more or less limited to additive gene transfer, *i.e.* genes can be introduced into the host genome, but no deletion is possible (Niemann, Kues, Petersen, & Carnwath, 2011; Petersen & Niemann, 2014).

Nevertheless, these techniques were successfully used to produce farm animals with agriculturally important transgenic traits. Typical agricultural applications included improved carcass composition, lactation performance, and wool production; as well as enhanced disease resistance and reduced environmental impact. With the advent of Site Directed Nucleases (see 3.3.1), microinjection in the cytoplasm has been successfully used to create animals with targeted genetic modifications (Murray & Maga, 2016).

Lentiviruses belong to the family of retroviruses that can infect non-dividing eukaryotic cells. Lentiviruses have been used as vectors for transgenic animal production, because they are efficient for the delivery of genes into oocytes and zygotes. Lentiviral mediated gene transfer in livestock generates unprecedentedly high yields of transgenic animals due to multiple integration events. However, this has the disadvantage that there is an increased probability of unwanted side effects such as oncogene activation or insertional mutagenesis.

Targeted genomic modifications in mammals require functional homologous recombination protocols. These are well established for many years in the laboratory mouse, but with very few exceptions, could not be successfully applied in livestock species. This is mainly due to the fact that genome sequencing was lagging behind that in the mouse and pluripotent embryonic stem cell (ESC) lines could not yet established. Targeted genetic modifications are preferred over random integration because the genetic locus at which the new gene is inserted is defined, and is thus associated with a more consistent expression pattern, and concomitantly the risk of insertional mutagenesis is avoided.

Unintended effects of ETGM in animals

As in plants, the random integration of the transgene during the use of ETGM can lead to insertional mutagenesis and can influence the expression of other genes. So far, no pleiotropic effects due to the use of ETGM have been reported in farm animals but cannot be excluded.

3.2.3. Microorganisms

In 1973 Herbert Boyer and Stanley Cohen created the first transgenic organism by inserting antibiotic resistance genes into the plasmid of an *Escherichia coli* bacterium. Taking a gene from a bacterium that provided resistance to the antibiotic kanamycin, they inserted it into a plasmid vector and then induced other bacteria to take up the plasmid. These bacteria were then able to survive in the presence of kanamycin (Russo, 2003).

In 1976 the first genetic engineering company, Genentech, was founded by Herbert Boyer and Robert Swanson and a year later the company produced a human protein (somatostatin, a growth hormone inhibiting hormone) in *Escherichia coli*.

DNA constructs produced *in vitro* are usually introduced into the microorganisms by various transformation protocols such as electroporation. Inside the recipient organism the DNA construct is subsequently stably maintained either by integration into the host genome or on a self-replicating plasmid thus producing a recombinant organism.

A much less frequently used method for transfer of DNA constructs is the introduction via conjugation from a donor organism such as *Escherichia coli*. Conjugation is particularly useful to overcome those cases where the recipient organism is resistant to transformation. Transduction can also be used to transfer DNA between closely related bacterial strains. Transduction played an important role in mapping, isolating and combining mutations in model bacteria such as *Escherichia coli* but nowadays has been largely surpassed by transformation for strain engineering. Finally, stable hybrids can be formed by protoplast fusion after enzymatically removing the cell walls of microorganisms. The methods differ to some extent but the strategy works both for bacteria and fungi including yeast (Hayat & Christias, 2010; Kitagaki & Kitamoto, 2013).

The most typical ETGM approach for generating genetically altered lactic acid bacteria (LAB) is electroporation with plasmid vectors. Alternative systems include conjugation and transduction. Targeted replacement of chromosomal genes or removal and inactivation of genes can be applied via non-replicating vectors using the natural event of crossing-over during cell division and DNA replication. Most of the food-grade integration vectors are designed to perform integration within non-coding regions on the chromosome, without affecting surrounding genes (Peterbauer, Maischberger, & Haltrich, 2011).

DNA constructs are introduced into yeast usually by transformation and introduced DNA molecules are either stably maintained as a

minichromosome (using an autonomous replication sequence ARS and centromere CEN) or as a multicopy plasmid (using the replicator of the endogenous yeast 2µ plasmid) or stably integrated into the genome by homologous recombination, which is very efficient in yeast in contrast to animals and plants.

The end products which can result from the employment of ETGM in microorganisms are split into three groups in European Food Safety Authority Guidance (EFSA, 2006). Those in the first group would be single compounds or defined mixtures of compounds derived from GMM, such as amino acids, vitamins or pure enzymes. Those in the second group would be complex products derived from GMM but not containing any viable organisms nor recombinant DNA, such as cell extracts, some feed enzymes, wine and some beers, etc. Those in the third group would be GMM and products which contain viable GMM or intact recombinant DNA and would include live or heat-killed starter cultures and probiotic cultures, some beers, cheeses, yoghurts, etc.

The large variety of end products generated using bacterial and fungal strains includes enzymes, for example milk-clotting enzymes, and food and feed additives, such as aspartame and L-lysine. (Blair & Regenstein, 2015), (Adrio & Demain, 2010).

Unintended effects of EGTM in microorganisms

The EGTM of microorganisms usually involve the precise introduction of mutations/genes via homologous recombination, which usually does not result in unintended DNA integration. The present level of WGS technologies allows for complete sequencing of microbial genomes, which are smaller and less complex than the ones of animals and plants. WGS can thus be used for confident confirmation of the intended changes introduced, as well as for detection of unintended integrations. Pleiotropic effects of the mutations introduced (see chapter 4) however cannot be excluded.

3.3. New breeding techniques (NBT)

Recent scientific progress has enabled the development of a new generation of techniques, which are often referred to as 'New Breeding Techniques' (NBT). The term NBT describes a very diverse range of techniques, some of which are substantially different from established transgenic approaches in their way of introducing traits to an organism (EASAC, 2015). Some are a refinement of CBT and insert genetic material that is derived from a sexually compatible species, while some nevertheless are used in combination with ETGM.

Some of the NBT result in organisms that contain only point mutations and are practically indistinguishable from varieties bred through CBT methods or resulting from spontaneous mutations (see chapter – 4 Comparisons). Whereas several NBT mentioned below are restricted to plants, others, in particular the recent techniques of genome editing, are applied in plants, animals and microorganisms (Lüthi et al., 2012; OECD, 2016).

3.3.1. Genome editing technologies

Genome editing aims to achieve a precise alteration of a DNA sequence in a cell, or to achieve random changes at precise locations. It is achieved with the aid of the cell's DNA recombination/repair system activated with the use of a site-directed nuclease *(SDN)*, exogenous nucleic acid molecule (oligonucleotide), or the combination of both (Royal Netherlands Academy of Arts and Sciences, 2016). A timeline of genome editing is shown in Figure 6.

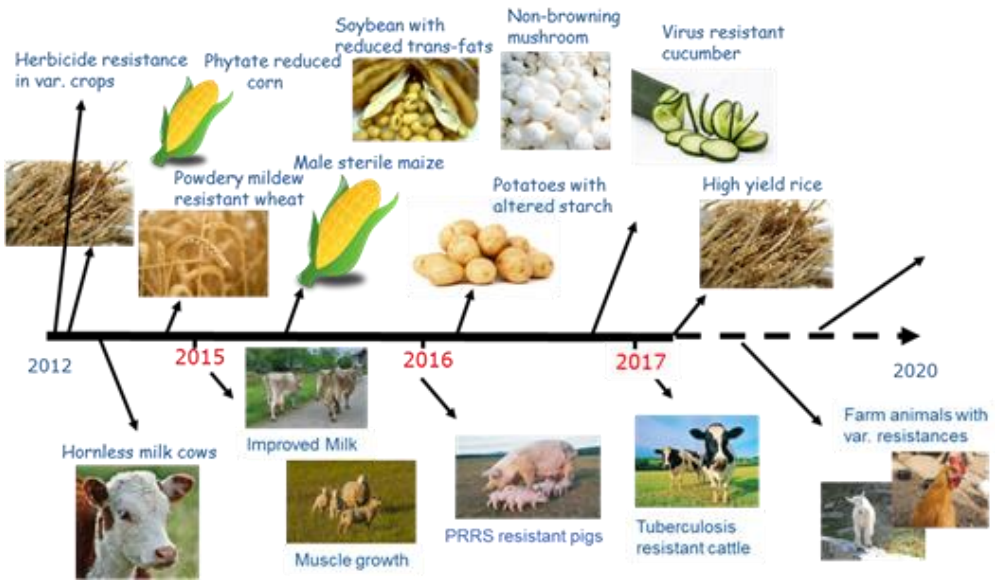

Figure 6 - Genome editing timeline- applications in crops and farm animals

Source: Author's

Oligonucleotide Directed Mutagenesis (ODM)

ODM is based on the use of oligonucleotides for the induction of targeted mutations in the genome, usually of one or a few adjacent nucleotides. The genetic changes that can be obtained using ODM include the introduction of a new mutation (replacement of one or a few base pairs, short deletion or insertion) or the reversal of an existing mutation.

The oligonucleotides employed are approximately 20 to 100 nucleotides long and are chemically synthesised in order to share sequence with the target DNA sequence in the host genome, with the exception of the nucleotide(s) to be modified.

Oligonucleotides can be delivered to cells by the common methods suitable for the different cell types (see *e.g.* 3.2.1).

Oligonucleotides bind to the complementary target sequence in the genome and this generates one or more mismatched base pairs corresponding to the non-complementary nucleotides. The cell's own DNA repair system

recognises these mismatches and removes them by using the sequence of either of the DNA strands (the oligonucleotide or the complementary target in the genome) to synthesize a second strand with a fully complementary sequence. If the mutagenic oligonucleotide is used as a repair template, its sequence is copied into the cell's DNA. As a result, a desired change in the target sequence in the genome is produced. The mutagenic oligonucleotides are degraded in the cell, but the induced mutations are stably inherited (Lusser, Parisi, Plan, & Rodríguez-Cerezo, 2011).

Because of the characteristics of this technique (no DNA cut, requirement of large oligonucleotides of more than 20 to 100 nucleotides in length, and low efficiency (maximally 0.05%), off target effects are not expected. However, no published data were found concerning the off-target rate for ODM.

The ODM technique can contribute to introduce natural allelic variations which could be also obtained by crossing, but in a more difficult and lengthy process. However site-directed nucleases and in particular SDN2 (see below) seem more adapted and flexible for this purpose.

Site-Directed Nucleases

Genome modification has become more widely applicable via the development of site-directed nucleases (SDNs) that cut DNA at selected target sites producing what are called double-stranded breaks (DSBs). The purpose in doing so is to enable the insertion of random (SDN1), or non-random (SDN2) mutations in precise locations, or to enable the insertion of large segments (such as genes) in precise locations (SDN3).

The three applications of SDN techniques rely on natural cellular mechanisms for repairing such cuts in DNA. SDN1 relies on the non-homologous end joining (NHEJ) pathway and SDN2 and SDN3 on homology-directed repair (HDR). Without such repair mechanisms, cells may face irreversible damage or death.

DSBs are repaired by either HDR or NHEJ. In nature, the HDR mechanism is preferable from a cellular integrity point of view, since HDR repairs the cut, and any degradation of base pairs adjacent to the cut, 'as good as new' by

using the identical sister chromatid as a template. However, when SDN are present, as precise repair would lead to restoration of the cut site, which would be broken again by the SDN, this process of break-repair goes on until imprecise repair occurs and leads to mutation of the DSB target site. NHEJ can result in precise repair of a DSB, but can also generate imprecise repair and introduce random nucleotide substitutions and small insertions or deletions. This may lead to gene knockout (*e.g.*, by causing a shift in the target gene's reading frame or by mutating a critical region of the encoded protein). This is why the NHEJ mechanism is considered 'error prone' and is elicited for the targeted insertion of random mutations (SDN1).

HDR uses a DNA molecule with sequence(s) corresponding to the target site as a template for repair (the sister chromatid in nature) and enables repair without errors. The HDR machinery can, however also use an exogenous homologous oligonucleotide which is intentionally introduced in the cell for the purposes of genome editing (SDN2 or SDN3).

In this way, HDR can be exploited for SDN2 or SDN3 to generate the desired sequence replacement at the DSB site through homologous recombination guided by a donor DNA template, causing targeted nucleotide substitution, deletion, or insertion, or more complex alterations. In the end this may lead to alteration of the gene's function, gene inactivation or gene correction (Symington & Gautier, 2011;Wang, La Russa, & Qi, 2016).

Early but limited success (Cong et al., 2013; Jinek et al., 2012; Schiml & Puchta, 2016) was achieved with protein-directed SDN*s* such as mega-nucleases, zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs). ZFNs and TALENs are proteins which are engineered to both recognise specific DNA sequences and to cut DNA in the region of such sequences. ZFNs and TALENs consist of a modular DNA binding domain, which recognises a specific DNA sequence, attached to a nuclease which cuts one of the DNA strands at the binding site. Acting in pairs of ZFNs and TALENs, these protein-directed SDNs create double strand breaks at the targeted sequence. ZFNs and TALENs are less popular

at present because of the considerable effort required to produce pairs of proteins for every editing procedure in comparison to the CRISPR/Cas (see below) system.

The techniques of genome editing have advanced rapidly following the development of RNA-directed SDNs based on the bacterial CRISPR (clustered regularly interspaced short palindromic repeats) system and CRISPR-associated (Cas) nucleases (Cong et al., 2013; Jinek et al., 2012; Schiml & Puchta, 2016).

RNA-directed SDNs consist of a protein module (nuclease) which is bound to a guide RNA, the sequence of which targets the nuclease to the complementary DNA sequence in the genome. They are much easier to produce than the corresponding ZFNs and TALENs (Symington & Gautier, 2011).

The ZFNs and TALENs have been used to edit plant and animal genomes (Hauschild et al., 2011; Zhang & Voytas, 2011). However, CRISPR–Cas has quickly become the technology of choice for most genome editing applications due to its simplicity, efficiency and versatility (Bortesi & Fischer, 2015; Doudna & Charpentier, 2014; Ma, Zhu, Chen, & Liu, 2016; R. Peng, Lin, & Li, 2016; Stout, Klaenhammer, & Barrangou, 2017).

Application of SDN in plants

The CRISPR/Cas9 system is used in plants to introduce genome modifications for precision crop trait improvement (Khatodia, Bhatotia, Passricha, Khurana, & Tuteja, 2016; Y. Zhang et al., 2016).

A common technique employed is the delivery of DNA constructs encoding CRISPR/Cas9 into plant cells by *Agrobacterium tumefaciens* mediated T-DNA transfer or biolistic bombardment (plasmid DNA) (see 3.2.1). These constructs are expressed, and the product of their expression (CRISPR-Cas9) leads to cleavage of target sites and produce mutations (Bortesi & Fischer, 2015).

During this process, CRISPR/Cas9 constructs are integrated into the plant genome (Zhang et al., 2016), but crossed out in the next generation. The

stable integration increases the chance of producing off-target effects (cleavage and mutation at unintended genomic sites similar but not identical in sequence to the desired site), because of persistent nuclease activity, an issue which concerns the use of CRISPR/Cas9 in plants, animals and microorganisms (Liang et al., 2017), and which is discussed below in 'Unintended effects of CRISPR/Cas and recent developments for further improvement'.

Besides SDN1 also SDN2 genome editing has been applied in plants and templated mutations have been obtained, though so far only with a low success rate (Li et al., 2013; Svitashev et al., 2015).

The simultaneous or staggered introduction of changes at several locations in the genome is also possible by using several different RNA guides. Simultaneous mutations in 14 genes have recently been introduced in *Arabidopsis thaliana* without the detection of off-target effects (Peterson et al., 2016). The simultaneous introduction of multiple changes in the plant genome with such precision and efficiency is not achievable with CBT or ETGM. The high efficiency of the CRISPR/Cas system also allows the simultaneous inactivation by SDN1 of all the alleles in polyploid plants such as hexaploid wheat (Liang et al., 2017; Wang et al., 2014) and for a tetraploid potato (Andersson et al., 2016; Peterson et al., 2016).

In the common white button mushroom (*Agaricus bisporus*) the employment of SDN has been reported to reduce browning (Waltz, 2016).

Application of SDN in farm animals

SDNs have emerged as valuable molecular tools that have the potential to revolutionise breeding of large animals. SDN-based techniques of genome editing have been rapidly employed in animals for agricultural and biomedical purposes. Plasmids carrying the specific DNA nuclease can either be injected into the cytoplasm of zygotes or transfected into a somatic cell that is subsequently transferred into an enucleated oocyte (see SCNT). The plasmid is usually not stably integrated into the host genome. Instead the genetic alteration is achieved by transient expression of the DNA nuclease.

Within the short time of availability, several prominent examples have already been reported for agricultural applications including the increase of disease resistance in pigs (Whitworth et al., 2015), and cattle (Gao et al., 2017; Wu et al., 2015); improved performance of cattle, pigs, sheep and goats (Crispo et al., 2015; Cyranoski, 2015; Guo et al., 2016; Proudfoot et al., 2015; Yu et al., 2016); production of allergen reduced or allergen free animal derived products, *e.g.* bovine milk (Yu et al., 2011) and chicken eggs (Oishi, Yoshii, Miyahara, Kagami, & Tagami, 2016); and for improving animal welfare (Carlson et al., 2016). A number of biomedical applications are also reported with indirect relevance to agricultural applications but are not listed here given the scope of this Note.

The use of DNA nucleases also enables mutagenesis of multiple targets in animal genomes. For instance, it has been demonstrated that the 62 copies of Porcine Endogenous Retroviruses (PERVs) in pigs can be knocked-out simultaneously (Yang et al., 2015). Contrastingly, CBT in animals enable multiple changes in the genome but only in a non-specific and time-consuming way. Similarly, with ETGM, multiple targeted changes are very difficult to achieve.

Application of SDN in microorganisms

The activity of CRISPR-Cas has first been demonstrated in bacteria, and CRISPR-Cas systems and tools have been used in food bacteria, in particular for starter cultures and probiotics, encompassing strain-typing, phage resistance, plasmid vaccination, genome editing, and antimicrobial activity (Stout et al., 2017) and also in yeast.

Unintended effects of CRISPR/Cas and recent developments for further improvement (in plants, animals and microorganisms)

To exploit the full potential of genome editing, important questions and challenges must be addressed (Doyle et al., 2012; Hsu et al., 2013).

Although CRISPR/Cas seems to show the greatest potential and flexibility for genome editing, sequence requirements within the protospacer adjacent motif (PAM), which is necessary for target recognition, may constrain some

applications. In the meantime many related systems have been discovered, which use different PAM sequences, and these are increasingly being employed for genome editing (*e.g.* (Kleinstiver et al., 2016; Slaymaker et al., 2016).

Off-target mutations of genome-edited plants are a matter of concern, described for instance in the GenØk Biosafety Report of 2015 (Agapito-Tenfen, S.Z. & Wikmark, 2015), although this is much less an issue than with classical mutagenesis (mutation breeding, see 3.1.1 and 3.1.3). Much progress has been made since 2015 for closing the knowledge gaps regarding the mechanisms of DNA repair employed by genome editing techniques and for increasing the efficiency and precision of these techniques, which is explained in the following paragraphs.

Additional studies will, however, be required to evaluate the specificity and toxicity of RNA-guided DNA endonucleases *in vitro* and *in vivo*. This is especially important when their use is considered in animals in the future. For plants and microorganisms it will suffice to select the individual with the required changes in the genome from the pool of treated organisms. WGS can be used to check for the presence of off-target mutations with the restriction that in animal and plant genomes not all changes might be detected due to some technical limitations (*e.g.* there might be sequencing errors and some regions of the genome such as highly repetitive sequences are difficult to sequence). The detection of off-target effects of SDN by PCR and WGS is greatly facilitated by the analysis of specific candidate sequences corresponding or similar to the target for the nuclease.

Comprehensive profiling of off-target cleavage sites will provide insight into the stringency of target recognition in each system, which in turn will help to increase the specificity of the systems and to develop algorithms that calculate the most promising sequences to be targeted within a specific locus (Doyle et al., 2012; Hsu et al., 2013).

Off-target effects are exacerbated by excessive or prolonged Cas9 activity (Hsu et al., 2013; Pattanayak et al., 2013). When zygotic injections of CRISPR-Cas9 constructs are used to generate mutant animals, or plant cells

are transformed with such constructs, Cas9 activity after the initial rounds of mitosis can give rise to **mosaic genotypes** (Yen et al., 2014). Mosaicism is due to the creation of multiple alleles. It refers to the heterogeneity of tissues in one and the same organism including in this case tissues from cells in which the genome was edited and wild-type tissues.

Multiples alleles can result from SDN1 genome editing when two alleles of a homozygote plant have been mutated in a different way through random events. The two alleles can be effectively mutated but slightly differ from the molecular point of view. A strict identity of the two alleles can be obtained in the progeny through Mendelian segregation of the two alleles and the selection of the organisms bearing the two identical alleles.

The '**filler DNA mechanism**' is a phenomenon which also occurs frequently in nature when spontaneous DNA breaks are repaired by NHEJ using neighbouring or distant sequences (often from mitochondrial or chloroplast origin) (Gorbunova & Levy, 1997; Kim et al., 2016). It has been attributed to the action of a specific polymerase in the cell: PolQ (van Kregten et al., 2016). Exogenously added DNA including introduced CRISPR/Cas constructs may also be used for such repair and become inserted either completely or after partial degradation into intended and/or unintended genomic sites. If such exogenous DNA is detected, the organism will not be used as the end product.

A major challenge is to **improve the efficiency of genome editing** with a template via HDR (SDN2, SDN3) **while reducing unintended insertions and deletions** (indel) generation through NHEJ (SDN1). In fact it has been found that this is controlled to some extent at the level of the cell cycle: in the G1 phase NHEJ and thus SDN1 are effective, but not HDR and thus SDN2 or SDN3 (Orthwein et al., 2015). The Cas9 nickase has been developed, which cuts only one of the DNA strands instead of both, favouring HDR in the cell cycle during DNA replication, while preventing NHEJ repair during the G1 phase (Schiml, Fauser, & Puchta, 2014).

Various other experimental approaches are being developed including altering the HDR:NHEJ ratio by the use of small chemical molecules or by inhibition of NHEJ (van Kregten et al., 2016; Wang et al., 2016).

The incidence of off-target mutations is mainly dependent upon the uniqueness and length of the recognition site including the essential PAM sequence, and also upon structural context of the recognition site in the genome (chromatin structure), the composition and structure of guide RNA, and the duration of the treatment with an SDN (Barakate & Stephens, 2016; Xue et al., 2015).

Substantial efforts are being made to improve CRISPR/Cas9 mediated genome editing with the aim of avoiding transgene integration and off-target mutations. Transgene integration can be avoided by transformation with the mRNA encoding CRISPR/Cas9 or the purified CRISPR/Cas ribonucleoprotein (RNP) complex. At the same time this also limits the duration of nuclease activity and thus reduces off target effects. Recently, Zhang et al., (2016) showed that transformation of wheat with CRISPR/Cas9 mRNA (TECCRNA) resulted in efficient genome editing. Woo et al., (2015) demonstrated that preassembled CRISPR/Cas9 ribonucleoproteins (RNPs) complexes can be used for genome editing and greatly decreased off-target mutations. The authors delivered the RNPs into lettuce protoplasts and obtained transgene-free mutant plants. A disadvantage is that this requires protoplast and tissue culture procedures which are not available for all plants and may lead to somaclonal variation (see text box 2).

Svitashev and co-authors (Svitashev, Schwartz, Lenderts, Young, & Cigan, 2016) and (Liang et al., 2017) also reported targeted mutagenesis in maize without integrated transgenes using the CRISPR/Cas9 ribonucleoprotein complexes. These studies suggest that the use of RNPs CRISPR/Cas9 RNPs has the potential to substantially aid specific and precise genome editing in a wide range of plant species.

Methods to activate CRISPR/Cas9 genome editing in response to specific cues have been devised, including light-inducible and drug-

inducible Cas9 activity (Nunez, Harrington, & Doudna, 2016). Very recently, genetically encodable "off-switches" for Cas9 activity have been identified that can be used as inhibitors of genome editing (Maxwell, 2016; Puchta, 2016).

The genome size of microorganisms is much smaller than that of plants and animals. Because of this, the probability of off-target effects is much lower as the existence of the same target sequence in the microbial genome is less likely (Stella & Montoya, 2016). SDN1 is not effective in many bacteria because of a lack of NHEJ activity. SDN2 and 3 are possible, but usually combined with "recombineering". Recombineering was developed for targeted mutagenesis using a template ssDNA in *E.coli* and employs the use of the ssDNA binding protein from phage λ or RecT for another phage, but has also been developed for LAB (Van Pijkeren & Britton, 2012).

In general, the probability of unintended effects on the expression of other genes as well as pleiotropic effects due to genome editing with CRISPR/Cas9 related methods is low because the targeted sequence is known.

Applications of engineered SDNs for regulation of gene expression

A nuclease-deficient Cas9, termed dCas9, has been developed, which maintains the ability to bind both the guide RNA and targeted DNA, but it does not cleave the DNA. dCas9 has been thus used as a sequence-specific RNA-guided DNA-binding platform for the development of new tools for engineering the genome as well as for rewriting its epigenetic status in a sequence-specific manner. dCas9 has been fused to transcriptional repressor or activator domains, which leads to suppression or upregulation, respectively, of target genes without changing their sequence or epigenetic status. Similarly, DNA binding domains such as ZFs and TALE domains have been linked to repressor or activator domains to affect gene expression (Lindhout, Pinas, Hooykaas, & Van Der Zaal, 2006). Independently, the guide RNA can be also engineered to be used as a scaffold to recruit regulatory molecules (endogeneous or exogeneous). When different regulatory molecules are coupled to different guide RNAs, different genes

can be regulated at the same time in different ways (*e.g.,* some repressed, some activated).

Engineered nuclease-deficient SDNs (protein-directed and RNA-directed ones) can be also used to introduce or remove epigenetic modifications, including DNA methylations and histone modifications (methylation and acetylation). By fusing epigenetic modifying enzymes to dCas9, epigenetic modifications have been introduced that result in repression or activation of targeted genes.

Although most current applications of Cas9 make use of its sequence-specific DNA editing and targeting capabilities, some authors (Collonnier et al., 2015; Liu, Yuan, & Stewart Jr., 2013; Podevin at al., 2013) have suggested using SDNs to target RNA sequences and alter gene expression at post-transcriptional level, without interfering with the genome. The potential of using engineered SDNs to target RNA is considered a strong inspiration to further develop new applications of *e.g.* Cas9-based tools for various RNA modifications, such as to regulate the stability, localization, and splicing of the targeted RNAs (Dominguez, Lim, & Qi, 2015; H. Wang et al., 2016).

3.3.2. Techniques introducing genetic material from same or sexually compatible species: cisgenesis and intragenesis

As opposed to transgenesis, which can be used to insert genes from any organism, in cisgenesis and intragenesis, only the gene pool of the recipient species and/or of sexually compatible species is used as a source for the genetic construct to be inserted. Sexually compatible species may be a closely related cultivated species or related wild species. In the case of cisgenesis, the entire gene with its own regulatory elements is inserted. In the case of intragenesis, the inserted DNA can be a new combination of regulatory or coding DNA fragments from the species itself or from a cross-compatible species.

Cisgenic and intragenic crop plants are generated by similar methods of gene transfer as used in transgenesis, however, without the use of marker genes (see section 3.2), predominantly by *Agrobacterium*-mediated

transformation and biolistic transformation (VIB, 2016). In principle, products similar to cisgenic products, but not to intragenic products, could be obtained by conventional breeding, although the location of the inserted gene would differ for each cisgenic organism due to random insertion and position effects would therefore be different for each event.

Cisgenesis and intragenesis can be applied for example in crops which are difficult to breed and into which it is difficult and time consuming to introduce other alleles from the gene pool. For example, three different *Phytophthora* sp. resistance genes have been introduced into a commercial potato cultivar within a few years by cisgenesis. Other examples are scab-resistant apple, into which genes conferring resistance to the fungus *Venturia inaequalis*, in particular from the Vf gene originated from Malus floribunda have been introduced. Previously, the introduction of a single resistance gene took almost 50 years through conventional breeding (Baltes & Voytas, 2015; Krens et al., 2015; Ryffel, 2017; Schaart, van de Wiel, Lotz, & Smulders, 2016).

The cisgenic *Phytophthora*-resistant potato is under evaluation in field trials and could be commercialised in 5-10 years (Jan Schaart, Marleen Riemens, Clemens van de Wiel, Bert Lotz, 2015; Schaart et al., 2016). The combination of cisgenesis/intragenesis with genome editing techniques is also under investigation in order to combine the advantages of both approaches (similarity with conventional crossing and accuracy, respectively) (Cardi, 2016; Krens et al., 2015; van de Wiel, Smulder, Visser, & Schaart, 2016).

3.3.3. Agro-infiltration

Plant tissues, mostly leaves, are infiltrated with a liquid suspension of *Agrobacterium sp*. containing the desired gene(s) to be expressed in the plant. The genes are locally and transiently expressed at high levels.

Agro-infiltration can be used to screen for plants with valuable phenotypes that can then be used in breeding programmes. For instance, agro-infiltration with specific genes from pathogens can be used to evaluate plant resistance. The resistant plants identified in the agro-infiltration test might

then be used directly as parents for breeding. The progenies obtained will not be transgenic as genes may only be integrated in the somatic cells of the infected plant (for instance, a leaf). Movement of *Agrobacterium* through the plant cannot be fully excluded and therefore absence of transgenes needs to be checked by WGS in the progeny. Alternatively, other stored plants which are genetically identical to the identified candidate plant may be used as parents.

3.3.4. Epigenetic modification: RNA-dependent DNA methylation

RNA-dependent DNA methylation (RdDM) allows breeders to produce plants with desired traits that do not contain foreign DNA sequences and in which no changes are made in the nucleotide sequence of the genome. The technique relies on a natural process of enzymatic addition of a small chemical group (*e.g.* methyl) to nucleotides, which can be maintained in the methylated state in daughter cells following cell division (hence the name 'epigenetic').

RdDM regulates the expression of genes by the addition of markers or tags to the control regions of genes, while not changing the gene sequence itself. It can be used to 'switch off' genes, which would otherwise interfere with the expression of desired traits and 'lock' them in an 'off' state.

The technique usually relies on the production of intermediate transgenic plants. Foreign genetic material is introduced to these plants to induce gene silencing by the production of an antisense RNA molecule. This molecule binds to the mRNA expressed by the target gene. These double-stranded RNA molecules in turn trigger the formation of small non-coding RNA (sRNA) molecules in the cell, which cause transcriptional gene silencing by DNA methylation. This methylation pattern and thus the desired trait can be maintained for a number of generations following the elimination of the inserted genes.

Recently purified sRNA molecules have been used to induce gene silencing in plants and in insect pests after spraying on plant leaves, thus avoiding the introduction of recombinant DNA molecules altogether.

3.3.5. Grafting

Grafting is a frequently-used technique in which the stem of one plant species (scion) or variety is grafted onto the rootstock of another species or variety. This technique is almost standard in horticulture and tree nursery. As such this technique is not new at all. However, a more recent application of this technique is the grafting of a non-ETGM scion onto an ETGM rootstock. In this case the DNA of the scion that includes the fruits for harvest is not modified. Nonetheless, there is some exchange of the rootstock with the scion. Alongside water, sugars and other metabolites, small molecules (such as sRNA molecules) derived from the GM rootstock can be transferred.

A GM plant that is used as a rootstock can be developed to silence the expression of one or more genes. This often occurs through the production of sRNA molecules. These molecules can be transported to the scion where they can influence the expression of specific genes in an identical manner. So even though the DNA of the scion is unchanged, the production of certain proteins in the scion can still be adjusted by the rootstock (VIB, 2016). As above, plants in future may be protected against pests by spraying them with sRNA molecules rather than using transgenic plant rootstock or tissue producing the sRNA molecules (Gan et al., 2010; Ryffel, 2017; San Miguel & Scott, 2016).

3.3.6. Reverse breeding

Reverse breeding (RB), Figure 7, is a plant breeding technique designed to directly produce parental lines for any heterozygous plant, one of the most sought after goals in plant breeding (because it enables the maintenance of a plant in a "stable" state which can otherwise be lost due to meiotic recombination). RB generates perfectly complementary homozygous parental lines through engineered meiosis. The method is based on reducing genetic recombination by silencing (through RdDM) a key recombination gene in the selected heterozygote thus eliminating meiotic crossing over. Male or female spores obtained from such plants contain combinations of non-recombinant parental chromosomes which can be

cultured *in vitro* to generate homozygous doubled haploid plants (DHs). From these DHs, complementary parents can be selected and used to reconstitute the heterozygote in perpetuity (Dirks et al., 2009). The intermediate transgenic plant sequences employed to supress meiosis are backcrossed out to generate null segregants free of transgenic sequences.

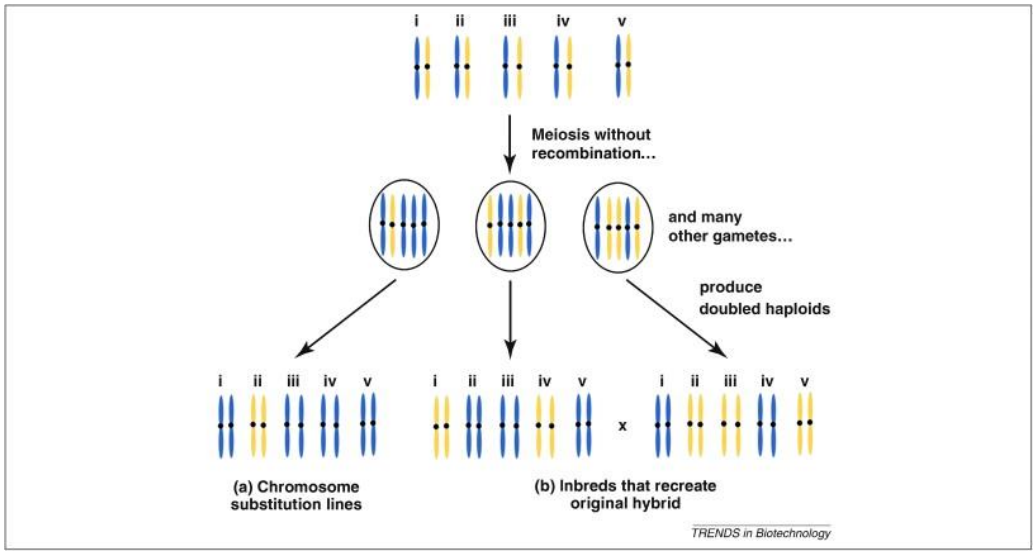

Figure 7 - Reverse breeding

Source:(Chan, 2010)

3.4. Application of NBT in gene drives and in synthetic biology

3.4.1. Synthetic biology

Synthetic biology is an emerging discipline and rapidly evolving technology that enables more powerful genetic design than previously.

Synthetic Biology combines a number of modern techniques from biotechnology, computer science and other areas to engineer new organisms that do not occur in nature and which can function *e.g.* as platforms for the biochemical production of products with economic relevance. However, the application of such techniques as well as other new bio-techniques or nanotechnology does not necessarily cause an organism or product to be of synthetic origin (Scientific Committee (SCHER/SCENIHR/SCCS), 2015).

When compared to modern biotechnology (*e.g.* ETGM) the novelty of synthetic biology lies in the systematic use of engineering approaches to intentionally design artificial organisms (Raimbault, Cointet, & Joly, 2016). In this way, large sets of genes encoding complete biochemical pathways from one organism are introduced in a new production organism. For instance the entire gene set determining the synthesis of complex metabolites discovered in a rare plant in nature can be identified, redesigned, adapted (for instance in codon usage and regulatory domains) and introduced for production in an industrial synthetic organism, such as a yeast or a fungus.

Thanks to the technological level reached by genetic engineering and the current knowledge regarding complete genomes sequences, large functional DNA molecules can now be synthesised efficiently and quickly without using any natural template (Lusser et al., 2011).

This is of particular interest when the metabolites cannot be chemically synthesised. The classic example of adding a metabolic pathway is in Golden Rice with increased levels of pro-vitamin A, which in its improved version has a transgenic construct of a phytoene synthase from daffodil combined with the originally used carotene desaturase from *Erwinia uredovora* (Paine et al., 2005). More extensive adaptation of pathways has been described for maize by Zhu et al., (2008) and Naqvi et al. (2009, 2011). They used combinatorial transformation to enhance vitamin production in endosperm, which meant introducing 5 transgenic constructs, including various endosperm-specific promoters, simultaneously through a biolistics approach followed by selecting plants expressing several or the complete set of transgenes.

Besides complex biochemical pathways, entire genomes can now also be redesigned and synthesised. In 2010, scientists at the J. Craig Venter Institute created the first living organism with a synthetic genome by transplanting a chemically synthesised genome (a modified genome of bacterium *Mycoplasma mycoides* that occurs naturally) to cells of another bacterium *Mycoplasma capricolum*, the original genome of which had been

destroyed in the process. Recently, yeast strains were similarly developed with a number of synthetic yeast chromosomes.

Synthetic genomics not only provides the possibility to reproduce existing pathways and organisms *in vitro*, but the synthesis of building blocks enables the creation of modified natural or even completely artificial pathways and ultimately organisms (Raimbault et al., 2016) for which no characteristic reference organisms exist in nature (Epstein & Vermeire, 2016).

To realise the full potential of plant synthetic biology, techniques are required that provide control over the genetic code – enabling targeted modifications to DNA sequences within living plant cells. Such control is now possible due to the latest advances and recently synthetic biology is benefiting from the application of genome editing (Baltes & Voytas, 2015).

For example, the ability to site-specifically integrate DNA into plant genomes will be of particular value for synthetic biology projects that require the transfer of numerous genetic parts to confer a novel biological function (Baltes & Voytas, 2015).

Challenges in assessing synthetic biology are foreseeable but the existing methods of risk assessment for GMOs and chemicals are applicable; however, new and rapid synthetic biology developments may require adapting existing methods for risk and safety assessment (Scientific Committee (SCHER/SCENIHR/SCCS), 2015).

3.4.2. Gene drives

During sexual reproduction of diploid organisms, each of the two alleles of a gene present in each parent has a 50% chance of being inherited by offspring. Gene drives change this probability and promote the inheritance of a particular gene to increase its prevalence in a population. In some cases, this inheritance is detrimental to the fitness of the organism. A wide variety of gene drives occur in nature with scientists first suggesting their use for the control of disease vectors (such as mosquitos) in the 1960s. Only with the development of the CRISPR-Cas9 system has a "precise and

predictable mechanism to cause the preferential increase in an existing or engineered trait" become possible (Committee on Gene Drive Research in Non-Human Organisms, 2016).

CRISPR-Cas9 mediated gene drive could theoretically spread a targeted gene through nearly 100% of a given population of sexually reproducing organisms, though the effect and efficacy of gene drives are likely to be species-dependent (Committee on Gene Drive Research in Non-Human Organisms, 2016).

At the molecular level, CRISPR-Cas9 gene drives work by introducing DNA encoding the CRISPR-Cas9 system and the desired gene into a chromosome. When transcribed, the system then cuts the homologous chromosome and the modified chromosome is used as a template for its repair. This results in two copies of the gene drive in the organism's genome, enables the transfer of the gene drive into offspring and its propagation through populations.

Potential agricultural applications (Committee on Gene Drive Research in Non-Human Organisms, 2016) include:

- Control or alter organisms that damage crops or carry crop diseases, *e.g.* fruit flies or aphids;
- Eliminate weedy plants that compete with cultivated crops;
- Eliminate herbicide or pesticide resistance;
- Control or alter organisms that carry infectious diseases that infect farm animals or organisms that directly cause diseases;
- Control or alter organisms that serve as reservoirs of farm animal diseases.

There are other specific issues described in (European Academies Science Advisory Council (EASAC), 2017) that could hinder the efficacy of a gene drive system in some populations.

In addition to efficacy questions, the increased interest in gene drives has led to questions about the potential safety of the technology. Discussions

have largely focused on two topics: the risks posed by using a technology that is self-sustaining, and the risks linked to an accidental release of gene drive organisms. Given the variety of ways gene drives could be applied, safety concerns need to be related to a specific product and cannot be realistically assessed in general terms (European Academies Science Advisory Council (EASAC), 2017).

In their review of gene drive opportunities and challenges the US National Academies of Sciences, Engineering and Medicine noted that it is essential to continue research to establish the efficacy and safety of gene drives before it can be decided whether they are suitable for use. The report concluded that the significant potential of this application justifies proceeding with phased research and testing so that benefits and risks could be properly assessed (National Academies, 2016).

Comparisons

4. COMPARISONS

4.1. General comments

In this chapter the NBT described above will be compared to (1) CBT and (2) ETGM. The SAM HLG has been asked to identify possible differences and similarities of these techniques in terms of: safety for health and environment, maturity for field applications, detectability of the respective products as well as speed and costs to achieve the expected result.

The comparisons presented here focus on the most relevant techniques, i.e. the techniques which are most commonly used for agricultural applications in plants, animals and microorganisms.

Tables 1A to 7A compare NBT with CBT, and tables 1B to 7B, NBT to ETGM. Only those techniques which are closest regarding the desired introduced changes and/or the molecular mechanism are compared however. Comparisons which are not made are listed 'not considered relevant' because of the differences in the desired changes or in the molecular mechanisms. Considering the molecular mechanisms, the NBT are a very heterogeneous set of techniques which can be used to develop a wide range of different products. It is also noteworthy that often the NBT are used in combination with other NBT as well as with CBT and ETGM.

Safety

The Note does not provide statements on the absolute or comparative safety of the different breeding techniques, as it is not possible to provide a scientifically sound safety assessment of breeding techniques as such. The safety assessment can evaluate the properties of each specific end-product only on a case-by-case basis. As described in the Environment Agency Austria report on new plant breeding techniques: "It is evident that a comprehensive evaluation of the potential risks of a specific NPBT[19]-crop cannot be based solely on generic considerations addressing only characteristics of the techniques or combination of techniques used to

[19] New Plant Breeding Technologies, *i.e.* new breeding techniques applicable for plants as described in section 3.3

generate the respective NPBT-crop. Rather a case specific approach is considered appropriate for the assessment of NPBT-crops." (Eckerstorfer, 2014).

Moreover, carrying out risk assessments of food and feed products is the responsibility of specific science advice structures at national and EU level. For instance, in 2012 the EFSA Panel on GMOs adopted two scientific opinions on the safety assessment of plants developed using three different NBT, namely cisgenesis, intragenesis as well as Zinc Finger Nuclease 3 and other Site-Directed Nucleases (EFSA Panel on Genetically Modified Organisms, 2012). Both for plants generated with cisgenesis/ intragenesis as well as for plants generated with the help of site-directed nucleases, the EFSA GMO Panel concluded that the 'Guidance for risk assessment of food and feed from GM plants' and the 'Guidance on the environmental risk assessment of GM plants' are applicable and do not need to be developed further. The EFSA GMO panel also concluded that 'it can be envisaged that on a case-by-case basis lesser amounts of event-specific data are needed for the risk assessment'.

Regarding the NBT of cisgenesis and intragenesis the Panel concluded 'that similar hazards can be associated with cisgenic and conventionally bred plants, while novel hazards can be associated with intragenic and transgenic plants.'

Concerning the site-directed nucleases the panel concluded that 'the SDN-3 technique can minimise hazards associated with the disruption of genes and/or regulatory elements in the recipient genome. Whilst the SDN-3 technique can induce off-target changes in the genome of the recipient plant these would be fewer than those occurring with most mutagenesis techniques. Furthermore, where such changes occur they would be of the same types as those produced by conventional breeding techniques'.

In general, the potential risks of a certain end product are a function of associated hazards and exposure factors. Possible hazards of end products of various breeding techniques depend on their specific features including the intended as well as unintended effects at genetic and phenotypic level.

Exposure is related to the specific application of the end product and the extent to which it is cultivated and consumed.

For example, for a plant with a new herbicide resistance trait, whether obtained through CBT or ETGM or NBT, the potential risk to human and animal health and/or the environment will depend on the sources and characteristics of the introduced genes, the specific trait, the species into which the trait is introduced, the environment in which it is cultivated, the applied agricultural practice (for example the correct use of herbicides) as well as the extent to which it is consumed (Andersson et al., 2012). For a review discussing various factors, which have to be considered in analysing the possible impact of GM crops as compared to conventional agriculture see for instance Barrows, Sexton, & Zilberman, (2014); Byravan, (2016); and European Commission, (2010).

Many NBT products could be obtained also by using CBT or ETGM albeit with less precision, less efficiency and, in particular in the case of the CBT, in a much more time-consuming way. Some products developed by NBT (*e.g.* targeted mutagenesis with SDN or targeted integration of exogenous DNA) are very specific and cannot be easily generated with other breeding techniques. Where the resulting phenotype and use are comparable, it follows that the risks would be similar too (Eckerstorfer, 2014).

Particular consideration must be given to unintended effects at genetic and phenotypic level that may appear with any type of breeding technique, including CBT. Unintended effects at genetic level may or may not manifest at phenotypic level and include for example the so-called off-target effects of SDN in genome editing (see below), undesired mutations induced through radiation or chemicals in conventional mutation breeding, unintended effects of sexual crosses or pleiotropic effects of transgenes in GM organisms (Ladics et al., 2015).

Table 2A and 2B contain information about the observed relative frequency of unintended effects related to the use of the different techniques. All breeding techniques can produce variable frequencies and severities of unintended effects. Their occurrence cannot be predicted and needs to be

assessed case by case. Independent of the breeding method, undesirable phenotypes are generally removed during selection and testing programmes by breeders (EFSA Panel on Genetically Modified Organisms, 2012).

It should be emphasised that 'unintended' does not necessarily mean 'harmful' and that the frequency of unintended effects thus does not allow for conclusions regarding safety (Ladics et al., 2015). In general, the precision of the NBT is expected to reduce some sources of unintended effects (Committee on Gene Drive Research in Non-Human Organisms, 2016). Research is ongoing to further reduce the frequency of unintended effects related to genome editing techniques.

In the case of plants, many CBT, ETGM as well as NBT, work with semi-differentiated plant tissues cultured *in vitro*, which induce somaclonal variations (spontaneous genetic and epigenetic changes), which are an important source of unintended effects (see text box 2 and paragraphs on 'unintended effects' in chapter 3).

All this has to be considered against the background of spontaneously occurring mutations and a broad range of unintended effects occurring during the application of any breeding technique (see page 21/ 22 on unintended effects and box 1).

Detectability/ Identification

The result of any breeding technique is an organism in which either the genome or its expression have been altered in some way, whether stably or transiently, locally or systematically and that may or may not – as a consequence - have an altered phenotype.

Detection

"Detection" in its broad sense therefore consists in investigating the difference between the original and altered organism by employing an analytical technology that specifically targets the changed molecule(s) (DNA, RNA, protein, …) or that scores the changed phenotype (*e.g.* change of flower colour). Two different scenarios for the detection of these changes

can be distinguished: (1) there is prior knowledge of the intended modifications (*e.g.* based on the information provided by the developer/the company together with the request for authorisation; (2) there is no prior knowledge or indication (*e.g.* from databases of authorised organisms) about the intended changes. In the second situation, no targeted detection method can be developed.

The principle of detection and the applied technologies involved are universal, *i.e.* they are specific for a certain type of molecule (DNA, RNA, proteins etc.) irrespective of the organism studied (virus, bacterium, plant, animal etc.) and independent from the procedure applied to modify the organism.

Figure 8 gives an overview of the various analytes (types of molecules) that can be targeted and gives some examples of techniques that have been successfully applied.

For the detectability of changes in different types of molecules it is important to consider that:

- The different analytes targeted have a different stability and different sensitivity to chemical and physical stressors. DNA for example is relatively stable over time and relatively insensitive to heating or to pH fluctuations, whereas RNA and protein molecules are usually much more sensitive and readily undergo degradation, making them no longer detectable.
- The quantity of analytes found that can be isolated may vary according to the type of matrix (*e.g.* embryo and endosperm in seeds have different analyte compositions) and may be influenced by different factors, such as environmental effects. Therefore, the RNA, protein and metabolite content may vary from zero to rather high concentrations already dependent on the respective tissue and environmental factors.

A suitable detection strategy must always be evaluated on a case-by-case basis and in some cases it may prove difficult to set up a suitable approach.

In general and as previously described DNA can be modified in a variety of ways such as by insertion, substitution or deletion of sequences as well as methylation of regulatory sequences to change the expression level of certain genes. Each of these techniques presents different specific challenges for detection.

Analyte targeted	Technique	Selected references	Essential requirements
DNA	Qualitative or quantitative Polymerase Chain Reaction (PCR)	(Lipp, Brodman, Pietsch, Pauwels, & Anklam, 1999; C. Peng et al., 2016; Takabatake et al., 2014)	Targeted DNA sequence must be unique. Targeted sequence must match requirements for the design of adequate primers. Fragments amplified should be relatively short for the reaction to meet performance requirements.
	Loop-mediated isothermal amplification (LAMP)	(C. Wang et al., 2015; Zahradnik et al., 2014; Zhou et al., 2016)	Requires a complicated design of multiple primers, with the associated target sequence requirements.
	DNA microarrays (with prior or Ligase detection reaction (LDR) and/or PCR)	(Nagarajan & De Boer, 2003)	Requires a pre-amplification step using PCR due to limited sensitivity. Commercial systems need to be available for application in a routine diagnostic laboratory
	Southern blotting	(Hill, Melanson, & Wright, 1999)	Requires labelled probes complementary to the specific target sequences.
	Next-Generation Sequencing (massive parallel sequencing of a targeted DNA sequence)	(C. Liang et al., 2014; Willems et al., 2016)	Requires the necessary equipment (next-generation sequencer) and the appropriate bioinformatics infrastructure, tools, reference databases and expertise.
RNA	Transcriptome sequencing	(Tengs et al., 2009)	The target protein's mRNA should be expressed in the tested tissue. Requires the necessary equipment (next-generation sequencer) and the appropriate bioinformatics infrastructure, tools, reference databases and expertise.
Protein	Enzyme-linked immunosorbent assay (ELISA)	(Asensio, González, García, & Martín, 2008; Rogan et al., 1999; Stave, 2002)	Antibodies specific to the protein of interest should be available. The sample cannot be too processed as this would affect its protein content, integrity and confirmation. The target protein should be expressed in the tested tissue.
	Western Blots	(Rogan et al., 1999)	
	Immunochromatographic (lateral flow) strip tests	(Nascimento, Von Pinho, Von Pinho, & do Nascimento, 2012; Stave, 2002)	

Figure 8 – Techniques for the detection and identification of genetic alterations

Source: JRC

Modifications by insertions for example are characterised by two new sequences: those that are generated at both ends of the site of insertion, called junctions. These junction sequences may present unique targets to base the detection approach upon. If targeted sequences are within the inserted sequence and internal to the junctions, then those are not unique to the resulting organism since, by definition, they also can be found in the organism they have been derived from. Evidently, this does not apply when completely new and unique sequences have been generated.

Deletion mutations also generate new, unique sequences and thus create one junction region that may be targeted but exceptions may be possible, for example if the deletion was introduced in a high-repeat region, creating non-unique junction region.

Another consideration is that no reliable detection can be accomplished unless a validated protocol and a reference material (preferably certified) are available.

Moreover for the detection of changes the organism under investigation must be compared to a reference organism which is as similar as possible. The most appropriate reference genome is the one that is obtained from the organism which will be changed, immediately prior to the change being made, in order to reduce the incidence of spontaneous mutations to as low a level as possible.

Without any prior knowledge and without the possibility of making postulations about the introduced change(s) (*e.g.* from databases of existing authorized GMOs) detection becomes much more challenging (Holst-Jensen et al., 2012). In such cases, WGS is almost the only analytical method by which the change(s) can be detected with the restriction that not all changes might be detected due to some technical limitations (*e.g.* there might be sequencing errors and some regions of the genome such as highly repetitive sequences are difficult to sequence). In general, the larger and the more complex a certain genome is, the more difficult it is to do a complete WGS.

Identification of the technique

Figure 9 summarises the minimal length for a DNA fragment to be unique in an organism as a function of its total genome size. Below this minimal length it is not possible to attribute the fragment to a specific organism, i.e. it is not possible to decide from which specific organism (the organism under investigation or another organism) the fragment originates.

Organism	Typical genome length (base pairs)	Approximate minimal length (base pairs)
Bacterium (*E. coli*)	$4.6x10^6$	13-15
Yeast (*S. cerevisiae*)	$1.2x10^7$	14-17
Crops (Maize)	$2.1x10^9$	17-20
Animals (Mouse)	$2.8x10^9$	18-20
Human	$3x10^9$	18-20

Figure 9 - Minimal length for a fragment to be unique in an organism as a function of its total genome size

Source: JRC

Likewise, if a technology is used that changes at least (for example) five nucleotides, then such a small change can be detected (by *e.g.* sequencing technology) but it is impossible to attribute the introduced changes to a certain breeding technique or to a naturally occurring phenomenon (spontaneous mutation) (Lusser, Parisi, Plan, & Rodríguez-Cerezo, 2011).

Many products with very similar or identical features at genetic and phenotypic level can be obtained using different techniques (CBT, ETGM, NBT), albeit with different precision and efficiency, or occur through natural processes. Thus, without any prior knowledge it is generally impossible to decide solely with analytical methods whether a certain change has occurred naturally or by technological intervention, let alone to identify the breeding technique underlying this change (Tables 1A and 1B, see also the JRC study: Lusser, Parisi, Plan, & Rodríguez-Cerezo, 2011).

Speed, costs, efficiency and ease of use

The comparison tables only contain some general statements about the relative costs for product development. Quantitative and even qualitative data about development costs for different breeding techniques, which are publicly accessible, are very scarce. Regulatory costs are not taken into consideration although they represent a major factor in the case of ETGM (Kalaitzandonakes, Alston, & Bradford, 2007). Likewise, in comparing the time needed to generate a product with the different techniques, the possible time needed for regulatory approval is not considered, which may, however, take the bulk of the time. Closely linked to costs and speed is efficiency, also strongly influenced by the ease of use of a certain technique, which is also reflected in the tables 5A and 5B.

Maturity

A qualitative statement is given in tables 7A and 7B on how close products obtained with NBT are to field trials and beyond from a purely technical point of view.

4.2. Comparison of new techniques (NBT) and conventional breeding techniques (CBT)

The most widely used CBT are mutation breeding and sexual crossing in plants, induced mutagenesis, conjugation/transduction or transformation as well as protoplast fusion in microorganisms and breeding based on population genetics with the help of AI and ET in animals.

Tables 1A to 7A compare NBT with the above mentioned CBT.

The statements made in the tables are made from the point of view of the NBT, mentioned on the X-axis, in the light of the CBT mentioned on the Y-axis. Where applicable, comparative statements are made and where possible in a quantitative way, otherwise in a qualitative way. The tables also contain some general statements which apply to all breeding techniques. The reader should refer to the main body of the text for references supporting the statements made in the tables.

As conventional mutation breeding cannot, or is not regularly used to achieve the effects produced by some NBTs, these NBTs are excluded from the comparison with mutation breeding/induced mutagenesis, namely: SDN3 (insertion of DNA fragments), Cis- and intra-genesis (insertion of DNA fragments from the same or a sexually compatible species), agro-infiltration (local transient transgene expression), reverse breeding (engineered meiosis and intermediate transgene insertion) and RNA dependent DNA methylation (intermediate transgene insertion). Agro-infiltration and RNA-dependent DNA methylation are excluded from comparison with sexual crossing in plants for similar reasons, namely that sexual crosses cannot achieve a similar effect. In addition, the NBTs agro-infiltration and RNA dependent DNA methylation are not applied in animals or microorganisms. Also cisgenesis and intragenesis are currently not used in farm animals or microorganisms. Hence they are not compared to CBT in animals and microorganisms, respectively.

4.2.1. Detectability/Identification

Detection and identification of changes are possible with a variety of analytical methods, if prior information on the intended change(s) is

available, *i.e.* if there is a known target to search for (which is not the case in mutation breeding where changes are random and numerous). If no information regarding the introduced change(s) is available (*i.e.* no known target for which to search), detection of changes is more challenging. Detection might be possible with WGS, possibly in combination with other approaches, but only with a suitable reference genome for comparison. For such cases, identification of the technique underlying the detected changes and distinction from natural variation solely with analytical methods is generally not possible (Lusser et al., 2011).

Mutations introduced by genome editing techniques can be detected for instance with the Surveyor Nuclease Assay, and T7-Assay. Putative off-target mutations can be detected by the same assays at sequences similar to the target site at which off-target mutation may be suspected or by WGS, with the restriction that WGS might not detect all off-target mutations due to sequencing errors and the impossibility to sequence certain regions of the genome. However, the relatively high variability of the animal and plant genomes within species makes it impossible to differentiate off-target mutations from spontaneously occurring mutations.

4.2.2. Unintended effects

Random mutagenesis through physical or chemical factors in plants and microorganisms induces a very large number of mutations (*e.g.* at a rate 500-fold higher than that of spontaneous mutations in plants (Cooper et al., 2008; Jander et al., 2003; Till et al., 2007). Consequently, the breeder must undertake time consuming downstream selection in order to identify the desired traits essentially on the basis of the phenotype. This selection process does not exclude the presence of unidentified mutations in the new variety.

The use of the new techniques involving ODM and SDN implies a different strategy. In this case the number of mutations is greatly reduced by comparison with the above and is limited to one or a few predefined mutations and possibly some off-target mutations. However, the function of the target gene selected for a mutation must be known. This prerequisite

remains a limiting factor which will be progressively addressed through the increasing knowledge of gene functions, amongst others due to the rapid progress of genomic studies. Thus, the genomes of several domestic animals and crop plants have now been sequenced and annotated ("Ensembl genome browser," 2017). The number of domestic animals and crops of which a draft genome is available is steadily growing.

Sexual crossing in plants involves the mix, at random, of different alleles of both parental plants but is restricted to sexually compatible plants. In contrast, SDN strategies such as SDN2 or SDN3 can specifically modify an allele in a desired way (allele conversion) independently of sexual barriers.

Likewise, when applying the CBTs of conjugation, transduction/transformation or protoplast fusion in microorganisms alleles from hundreds or even thousands of donor genes are introduced into the recipient whereas only one or a few mutations are introduced in a targeted way when using the NBT of gene editing.

The genome editing techniques (belonging to the NBT) may be accompanied by few unintended effects (section 3.3, in particular 3.3.1). However, in general the genome editing techniques show a much lower number or complete lack of unintended mutations as compared to organisms (plants, animals, microorganisms) obtained via CBT, in particular when compared to mutation breeding/ induced mutagenesis. End products containing only the desired mutation(s) can be obtained much more quickly as compared with CBT through screening & selection. The absence of unintended, potentially detrimental effects can be checked by WGS with some technical limitations (see also detection/identification 4.1.1).

4.2.3. Presence of exogenous DNA

In plants and animals, unintended effects notwithstanding, the NBT genome editing techniques of ODM, SDN1 and SDN2 do not result in end products containing exogenous DNA and are comparable to the CBT of mutation breeding and sexual crosses (in which a mixture of DNA sequences from the

genomes of two sexually compatible plants results) in this respect. SDN3, cisgenesis and intragenesis by contrast intentionally produce end products containing exogenous nucleic acids.

The introduction of exogenous DNA that contains mobile elements (*e.g.* transposons) into a recipient organism can lead to genetic changes that can be extensive (one or more insertion events in various sites and various unpredictable genome rearrangements, for example resulting from recombinations between copies of the mobile element). This pertains mostly to the use of traditional techniques that involve transfer of entire genomes or large sections of them because in ETGM and NBT techniques, the introduction of mobile genetic elements is generally avoided.

Conjugation/transduction, or transformation and selection; and protoplast fusion and selection in microorganisms each result in the inclusion of exogenous DNA in the form of several transgenes.

4.2.4. End products – characteristics

In general, when ODM, SDN1 and SDN2 strategies are used the induced changes represent very limited modifications of a pre-existing gene in the edited genome. Typically, the function of this gene is well characterised and the new characteristics should be limited to the impact of a well-defined mutation/modification on a pre-existing function. SDN3, cisgenesis and intragenesis results in the presence of a new allele. This is in contrast to the numerous random mutations or new alleles in plants, animals and microorganisms resulting from the various conventional techniques.

With CBT in plants and animals it is very difficult to separate desired traits from undesired ones in the end product and the targeted transfer of genetic information between different species is not possible.

4.2.5. Ease of use/efficiency

Mutation breeding/induced mutagenesis in plants and microorganisms result in massive genetic variation including neutral, detrimental and some rare useful genetic changes.

To obtain a crop with only the desired new trait, detrimental changes are removed by backcrossing with the parental line. After many generations of backcrossing, crop plants that are similar to the parent but which have incorporated the selected trait are obtained. It must be noted that mutation breeding is also used in vegetatively propagated species (apple tree, several ornamentals for example) without further crosses in order to maintain the other characteristics of the cultivar.

The same holds true for sexual crosses between a recipient and a donor with a desirable trait in plants. They result in mixed genomes in which initially half of the genes (alleles) are derived from the donor. By repeated backcrossing the numbers of donor alleles is gradually diminished until the donor allele for the selected trait is accompanied by as few other donor genes as possible (and which are typically located in its vicinity on the same donor chromosome).

With genome editing, mutations can be introduced in a much more targeted way. Depending on the technique, either unspecified mutations (SDN1) or specific mutations (ODM, SDN2) can be rapidly introduced into a desired gene creating a desired donor allele in the recipient (allelic conversion) without crossing in any other donor alleles. New alleles can also be cloned first and then introduced into the genome by transgenesis or cisgenesis. In polyploid plant species such as wheat, all the homologous copies of a gene can be targeted at the same time so that traits can be obtained which could only be obtained with great difficulty (molecular detection among thousands of plants of each mutated copy and crosses in order to stack the different mutated copies) by traditional mutagenesis.

4.2.6. Speed and costs

Major factors determining the speed of obtaining the end product of a breeding technique include the characteristics of the desired trait (monogenic or complex regulated trait) and in the case of animals the generation interval that differs amongst farm animal species and amongst plant species. For instance, in farm animals the generation intervals range from 6 months in pigs to 3 years in cattle. With the NBT of genome editing,

well characterised gene edited pigs can be produced within 6 months and gene edited cattle within 12-13 months. The costs of using genome editing techniques may critically depend upon the licensing fees for the DNA nucleases.

In microorganisms, the difference between CBT and NBT relates to the time and costs used for the planning of the strategy for introducing targeted mutations as compared to the time used for carrying out effective screening procedures, which vary between cases depending *e.g.* on how easily one can screen for the desired mutation at a phenotypic level.

Generally speaking, the NBT are faster and cheaper than CBT.

4.2.7. Maturity

There are already a number of plants generated with NBT, which are close to or at the stage of field trials or which are already commercialised. These include amongst others an herbicide resistant rapeseed variety obtained with ODM, which has been commercialised in Canada in 2017, a soybean modified with the use of SDN2 for oil composition intended to be commercialized in 2018 as well as *Phytophthora*-resistant potato and scab-resistant apple which are in field trials.

Practical applications of the genome editing techniques in animals already exist for biomedical applications, in particular for the production of pharmaceutical proteins in milk (gene pharming: GTC Biotherapeutics, Pharming, Biosidus) and xenotransplantation, *i.e.* the production of porcine organs and tissue in human organ transplantation. Agricultural applications are now also rapidly emerging (see 3.3.1).

Proofs of concept for the application of genome editing techniques have also been obtained in bacteria and fungi (Stout et al., 2017).

List of the Tables

NBT compared with CBT

Table 1A - NBT compared with CBT - Detectability/Identification

<table>
<tr><th></th><th>NBT
CBT</th><th colspan="4">Genome editing*</th><th colspan="2">Techniques introducing genetic material from same or sexually compatible species</th><th>Transgenes in intermediate step</th></tr>
<tr><th></th><th></th><th>ODM</th><th>SDN1</th><th>SDN2</th><th>SDN3</th><th>Cisgenesis</th><th>Intragenesis</th><th>Reverse Breeding</th></tr>
<tr><td rowspan="2">Plants</td><td>Mutation Breeding</td><td colspan="3">Detection and identification of changes are possible with a variety of analytical methods, if prior information on the intended change(s) is available, i.e. if there is a known target to search for which is not the case in mutation breeding where changes are random and numerous. If no information regarding the introduced change(s) is available, i.e. no known target for which to search as in the case of mutation breeding, detection of changes is more challenging. It might be possible with whole genome sequencing (WGS), possibly in combination with other approaches, but only with a suitable reference genome for comparison. For such cases, also identification of the technique underlying the detected changes and distinction from natural variation solely with analytical methods is generally not possible. Thus, changes to DNA sequence introduced by ODM, SDN1 and SDN2 which are generally relatively minor, are indistinguishable from those that can occur by spontaneous mutation or by induced mutagenesis.</td><td colspan="3">Not considered relevant</td><td>Not considered relevant</td></tr>
<tr><td>Crossing</td><td colspan="3">Detection and identification of changes are possible with a variety of analytical methods, if prior information on the intended change(s) is available, i.e. if there is a known target to search for. If no information regarding the introduced change(s) is available (i.e. no known target for which to search), detection of changes is more challenging. In the case of plants obtained by crossing there is information about the intended introduced changes and thus a known target. However, in addition alleles of hundreds or even thousands of donor genes are introduced into the recipient plant unintentionally, and it is challenging to detect all of these unintended changes. It can be possible with WGS, and in combination with other approaches, but only with a suitable reference genome for comparison. By crossing, the same allele can be introduced by sexual crossing, as an allele produced in the case of ODM, SDN1, and SDN2, or introduced with SDN3 (if fragment from sexually compatible species is introduced), and with cisgenesis. Therefore, identification of the technique underlying the detected changes and distinction from natural variation solely with analytical methods is generally not possible</td><td colspan="3">See ODM, SDN1, SDN2 vs. Crossing (left).

In the case of intragenesis (insertion of new combination of sequences) and SDN3 (insertion of DNA fragment e.g. from sexually non-compatible species) detection is usually easier than for ODM, SDN1 and SDN2 as the change is larger.</td><td>Detection and identification are not possible as the end product of reverse breeding only has naturally occurring alleles.</td></tr>
<tr><td>Animals</td><td>Breeding</td><td colspan="3">Detection and identification of changes are possible with a variety of analytical methods, if prior information on the intended change(s) is available, i.e. if there is a known target to search for. If no information regarding the introduced change(s) is available (i.e. no known target for which to search), detection of changes is more challenging. In the case of breeding there is information about the intended introduced changes and thus a known target. However, in addition alleles of hundreds or even thousands of donor genes are introduced into the recipient animal unintentionally, and it is challenging to detect all of these unintended changes. It might be possible with WGS, possibly in combination with other approaches, but only with a suitable reference genome for comparison. For such cases, also identification of the technique underlying the detected changes and distinction from natural variation solely with analytical methods is generally not possible. For instance the same allele can be introduced by conventional breeding, as an allele produced in the case of ODM, SDN1, SDN2, and in the case of SDN3 (if fragment from sexually compatible species is introduced).</td><td colspan="2">See ODM, SDN1, SDN2 vs. Animal Conventional Breeding (left).

In the case of SDN3 (insertion of DNA fragment e.g. from sexually non-compatible species) detection is usually easier than for ODM, SDN1 and SDN2 as the change is larger. Depending on the new DNA fragment introduced, the same changes can be introduced by conventional breeding.</td><td colspan="2">Not considered relevant</td></tr>
<tr><td rowspan="3">Microorganisms</td><td>Induced Mutagenesis & Selection</td><td colspan="3" rowspan="3">Detection and identification of changes are possible with a variety of analytical methods, if prior information on the intended change(s) is available, i.e. if there is a known target to search for, which is not the case in induced mutagenesis and selection, where changes are random and numerous. If no information regarding the introduced change(s) is available, i.e. no known target for which to search as in the case of induced mutagenesis and selection, detection of changes is more challenging. In the case of conjugation alleles from several donor genes are introduced into the recipient and in protoplast fusion alleles from hundreds or even thousands of donor genes. Their detection might be possible with WGS, possibly in combination with other approaches, but only with a suitable reference genome for comparison. For such cases, also identification of the technique underlying the detected changes and distinction from natural variation solely with analytical methods is generally not possible. Changes to DNA sequence introduced by ODM, SDN1 and SDN2 are relatively minor and are typically undistinguishable from those that can occur by spontaneous mutation or by induced mutation, and from minor changes that can be introduced by other CBT approaches.</td><td colspan="3">Not considered relevant</td></tr>
<tr><td>Conjugation/ Transduction or Transformation & Selection</td><td colspan="2">Same as for ODM, SDN1, and SDN2 vs. Conjugation/

Transduction or transformation & selection in Microorganisms (left). However, as compared to ODM, SDN1 and SDN2, non-targeted detection is usually easier because the introduced modification is larger (insertion of a DNA fragment). The same alteration can be introduced by conjugation, transduction, or transformation.</td><td colspan="2">Not considered relevant</td></tr>
<tr><td>Protoplast Fusion & Selection</td><td colspan="2">Same as for ODM, SDN1, and SDN2 vs Protoplast fusion & selection (left). However, as compared to ODM, SDN1 and SDN2, non-targeted detection is easier because the introduced modification (insertion of a DNA fragment) is larger. The same alteration can be introduced by protoplast fusion.</td><td colspan="2">Not considered relevant</td></tr>
</table>

*Genome Editing: includes amongst others ZFNs, TALEN, CRISPR/Cas and meganucleases

Table 2A - NBT compared with CBT – Unintended Effects

<table>
<tr><th></th><th>NBT
CBT</th><th colspan="4">Genome editing*</th><th colspan="2">Techniques introducing genetic material from same or sexually compatible species</th><th>Techniques in which transgenes are introduced in an intermediate step</th></tr>
<tr><th></th><th></th><th>ODM</th><th>SDN1</th><th>SDN2</th><th>SDN3</th><th>Cisgenesis</th><th>Intragenesis</th><th>Reverse Breeding</th></tr>
<tr><td>General</td><td></td><td>Oligonucleotides used for inducing mutations are usually degraded and not integrated into the genome. However, the possibility of their integration into the genome (as an unintended effect) cannot be completely excluded.
The presence of unintended exogenous DNA integrated into the genome can be detected with appropriate analytical methods (e.g. PCR, WGS, for technical restrictions see section 4.1). If exogenous DNA is detected, the organism will not be used as the end product.</td><td>In the case of the introduction of transgene sequences providing stable or transient expression of SDN proteins and guide RNAs, these DNA sequences may be integrated into the genome.
The presence of unintended exogenous DNA integrated into the genome can be detected with appropriate analytical methods (e.g. PCR, WGS, for technical restrictions see section 4.1). If exogenous DNA is detected, the organism will not be used as the end product.</td><td>In the case of the introduction of transgene sequences providing stable or transient expression of SDN proteins and guide RNAs, these DNA sequences may be integrated into the genome (like in SDN1).
Oligonucleotides used for providing homologous recombination are usually degraded and not integrated into the genome. However, the possibility of their integration into the genome (as an unintended effect) cannot be completely excluded (like in ODM).
The presence of unintended exogenous DNA integrated into the genome can be detected with appropriate analytical methods (e.g. PCR, WGS, for technical restrictions see section 4.1). If exogenous DNA is detected, the organism will not be used as the end product.</td><td>In the case of the introduction of transgene sequences providing stable or transient expression of SDN proteins and guide RNAs, these DNA sequences may be integrated into the genome (like in SDN1).
The presence of unintended exogenous DNA integrated into the genome can be detected with appropriate analytical methods (e.g. PCR, WGS, for technical restrictions see section 4.1). If others than the intended exogenous DNA is detected, the organism will not be used as the end product.</td><td colspan="3">Not considered relevant</td></tr>
<tr><td rowspan="2">Plants</td><td>Mutation Breeding</td><td>Much lower number or complete lack of off-target effects (unintended mutations at other than the intended positions in the genome), unintended effects on the expression of other genes and consequently of pleiotropic effects (mutation in one gene unintentionally influencing two or more seemingly unrelated traits).
In cases of unintended integration of exogenous DNA, it can be removed within one generation by backcrossing.</td><td>Depending on the mode of delivery of the nuclease to the cell (see 3.2.1 unintended effects) much lower number or complete lack of unintended effects (off-target effects, unintended effects on the expression of other genes and consequently of pleiotropic effects).
Regardless of the mode of the delivery of the nuclease, large deletions and insertions at the target site may rarely occur (instead of the desired local change), but less frequently than with mutation breeding. In addition, the detection of-off target effects is greatly facilitated by the analysis of specific candidate sequences corresponding or similar to the target for the nuclease.
In cases of unintended integration of exogenous DNA, it can be removed within one generation by backcrossing.</td><td>Depending on the mode of delivery of the nuclease to the cell (see 3.2.1, unintended effects) much lower number or complete lack of unintended effects (off-target effects, unintended effects on the expression of other genes and consequently of pleiotropic effects).
Regardless of the mode of the delivery of the nuclease, large deletions and insertions at the target site may rarely occur, but even less frequently than with SDN1 because of the different molecular mechanism (HDR).
In cases of unintended integration of exogenous DNA, it can be removed within one generation by backcrossing.</td><td colspan="3">Not considered relevant</td></tr>
<tr><td>Crossing</td><td colspan="3">Lower number or complete lack of unintended effects (off-target effects, unintended effects on gene expression and consequently of pleiotropic effects) as only one gene variant is created in a targeted way.
In sexual crossing alleles of hundreds or even thousands of donor genes are introduced into the recipient.
In cases of unintended integration of exogenous DNA, it can be removed within one generation by backcrossing.</td><td>Lower number or complete lack of unintended effects, if only one or few new sequences are introduced in a targeted way.
In cases of unintended integration of exogenous DNA other than the intended, it can be removed within one generation by backcrossing.</td><td>As in SDN3 vs Crossing in Plants (left). Unintended position effects are however possible and more frequent than in sexual crosses because the new alleles are inserted in a non-targeted way.</td><td>As in SDN3 vs Crossing in Plants (left), as only one or few new artificially constructed alleles using DNA from the same species or a crossable species are introduced. Unintended position effects are however possible and more frequent than in sexual crosses because the new alleles are inserted in a non-targeted way.</td><td>The presence of a transgene in the selected offspring is very improbable but needs to be verified.</td></tr>
<tr><td>Animals</td><td>Breeding</td><td colspan="3">Lower number or complete lack of unintended effects (off-target effects, unintended effects on gene expression and consequently of pleiotropic effects) as only one gene variant is created in a targeted way.
In conventional breeding alleles of hundreds or even thousands of donor genes are introduced into the recipient, but pleiotropic effects occur with low frequency in CBT and can be used in the crossing strategy.</td><td>Lower number or complete lack of unintended effects, if only one or few new alleles are introduced in a targeted way.</td><td colspan="3">Not considered relevant</td></tr>
<tr><td rowspan="3">Microorganisms</td><td>Induced Mutagenesis & Selection</td><td>Much lower number or complete lack of unintended effects (off-target, unintended effects on the expression of other genes and consequently of pleiotropic effects), due to smaller genome size even less frequent than in plants.</td><td colspan="2">Depending on the mode of delivery of the nuclease to the cell (see 3.2.3, unintended effects) much lower number or complete lack of unintended effects (off-target, unintended effects on the expression of other genes and consequently of pleiotropic effects), due to smaller genome size even less frequent than in plants.</td><td colspan="4">Not considered relevant</td></tr>
<tr><td>Conjugation/ Transduction or Transformation & Selection</td><td>Much lower number or complete lack of unintended effects, as one or a few mutations are introduced in a targeted way, whereas in conjugation, alleles from several donor genes are introduced into the recipient.</td><td colspan="2" rowspan="2">Much lower number or complete lack of unintended effects, depending on the mode of delivery of the nuclease to the cell (3.2.3, unintended effects).</td><td rowspan="2">Lower number of unintended effects, as only one or a few specific gene(s) is/are introduced in a targeted way.</td><td colspan="3">Not considered relevant</td></tr>
<tr><td>Protoplast Fusion & Selection</td><td>Much lower number or complete lack of unintended effects, as one or a few mutations are introduced in a targeted way whereas in protoplast fusion alleles from hundreds or even thousands of donor genes are introduced into the recipient.</td><td colspan="3">Not considered relevant</td></tr>
</table>

*Genome Editing: includes amongst others ZFNs, TALEN, CRISPR/Cas and meganucleases

Table 3A - NBT compared with CBT – Presence of Exogenous DNA molecule

	NBT / CBT	Genome editing*				Techniques introducing genetic material from same or sexually compatible species		Techniques in which transgenes are introduced in an intermediate step
		ODM	SDN1	SDN2	SDN3	Cisgenesis	Intragenesis	Reverse Breeding
Plants	**Mutation Breeding**	No exogenous DNA** present in the end product. For potential presence of exogenous DNA as an unintended effect in an intermediate product - see the general remark in Table 2A. Such undesired exogenous DNA can be removed within one generation by backcrossing. In comparison, in mutation breeding no exogenous DNA is introduced during the process.			Not considered relevant			
	Crossing	See ODM/SDN1/SDN2 vs. Mutation Breeding in Plants (above). In comparison, in sexual crosses the end product contains a mixture of DNA sequences from the genomes of two different, sexually compatible plants.			Exogenous DNA in the form of a gene linked to the desired trait is intentionally inserted into the end product at a targeted genome position. In comparison, in sexual crosses the end product contains rather a mixture of DNA sequences from the genomes of two different, sexually compatible plants.	Exogenous DNA in the form of one or a few cisgenes (allele(s) from the same or a sexually compatible species) is present in the end product. In comparison, in sexual crosses the end product contains rather a mixture of DNA sequences from the genomes of two different, sexually compatible plants.	Exogenous DNA in the form of one or a few intragene(s) (artificially constructed alleles with sequences from the same or a sexually compatible species) is present. In comparison, in sexual crosses the end product contains rather a mixture of DNA sequences from the genomes of two different, sexually compatible plants.	A transgene is only temporarily present at an intermediary step (in the first generation) to interfere with meiotic recombination. The end product, the doubled haploid plants and their offspring do not contain exogenous DNA. In comparison, in sexual crosses the end product contains rather a mixture of DNA sequences from the genomes of two different, sexually compatible organisms.
Animals	**Breeding**	No exogenous DNA present in the end product. For potential presence of exogenous DNA as an unintended effect in an intermediate product - see the general remark in Table 2A. In comparison, in breeding the end product contains a mixture of DNA sequences from the genomes of two different, sexually compatible animals.			Exogenous DNA in the form of a gene linked to the desired trait is intentionally inserted into the end product at a targeted genome position. In comparison, in breeding the end product contains rather a mixture of DNA sequences from the genomes of two different, sexually compatible animals.	Not considered relevant		
Microorganisms	**Induced Mutagenesis & Selection**	No exogenous DNA present in the end product. For potential presence of exogenous DNA as an unintended effect in an intermediate product - see the general remark in Table 2A. In comparison, in induced mutagenesis no exogenous DNA is introduced during the process.			Not considered relevant			
	Conjugation/ Transduction or Transformation & Selection	See ODM/SDN1/SDN2 vs. Induced Mutagenesis & Selection in Microorganisms (above). In contrast, conjugation/ transduction/ transformation can introduce exogenous DNA in the form of several transgenes.			Exogenous DNA in the form of a gene linked to the desired trait is intentionally inserted into the end product at a targeted genome position. In contrast, conjugation/ transduction/ transformation can introduce exogenous DNA in the form of several transgenes.	Not considered relevant		
	Protoplast Fusion & Selection	See ODM/SDN1/SDN2 vs. Induced Mutagenesis & Selection in Microorganisms (above). In contrast, in protoplast fusion exogenous DNA in the form of numerous transgenes can be introduced.			See SDN3 vs. Conjugation/ Transduction or transformation & selection in Microorganisms (above). In contrast, in protoplast fusion exogenous DNA in the form of numerous transgenes can be introduced.	Not considered relevant		

*Genome Editing: includes amongst others ZFNs, TALEN, CRISPR/Cas and meganucleases

**For the purpose of this explanatory note the term exogenous DNA is defined as DNA originating outside the organism of concern or under investigation which can be introduced naturally or by technological intervention

Table 4A - NBT compared with CBT – End-products

	NBT / CBT	Genome editing*				Techniques introducing genetic material from same or sexually compatible species		Techniques in which transgenes are introduced in an intermediate step
		ODM	SDN1	SDN2	SDN3	Cisgenesis	Intragenesis	Reverse Breeding
Plants	**Mutation Breeding**	Plants without exogenous DNA and with only limited targeted changes of a specific gene, **in contrast** to the numerous random mutations in plants obtained by mutation breeding.			Not considered relevant			
	Crossing	Plants without exogenous DNA and with only limited targeted changes of a specific gene (allele), **in contrast** to the plants with additional alleles of many other genes (introducing gene variants in addition to the intended ones) throughout the genome obtained by sexual crosses.			Plants with a new allele (gene variant) inserted in a targeted way at a chosen position in the genome **in contrast** to the plants with additional alleles of many other genes (introducing gene variants in addition to the intended ones) throughout the genome obtained by sexual crosses.	Plants with one or a few new alleles from the same or a sexually compatible species **in contrast** to plants with additional alleles of many other genes (introducing gene variants in addition to the intended ones) obtained by sexual crosses.	Plants with one or a few new artificially constructed alleles with sequences from the same or a sexually compatible species **in contrast** to plants with additional naturally occurring alleles of many other genes (introducing gene variants in addition to the intended ones) obtained by sexual crosses.	The end product of a reverse breeding procedure is **not different** from the products of a conventional cross (doubled haploids).
Animals	**Breeding**	Animals without exogenous DNA and with limited, targeted change(s) of a specific gene **in contrast** to animals with alleles of many other genes (gene variants) throughout the genome obtained by conventional breeding.			Animals with a new allele (gene variant) inserted in a targeted way at a precise position in the genome **in contrast** to animals with alleles of many other genes (gene variants) throughout the genome obtained by conventional breeding.	Not considered relevant		
Microorganisms	**Induced Mutagenesis & Selection**	Microorganisms without exogenous DNA and with a genome sequence that is almost identical to the parental line - with the exception of some limited targeted changes - **in contrast** to strains obtained through induced mutagenesis, which may contain numerous additional changes.			Not considered relevant			
	Conjugation/ Transduction or Transformation & Selection	Microorganisms without exogenous DNA and with a genome sequence that is almost identical to the parental line - with the exception of some limited targeted changes - **in contrast** to conjugative/transduced/transformed strains, which may contain some new genes.			Microorganisms with a new allele (gene variant) inserted in a targeted way at a precise position in the genome **in contrast** to conjugative/ transduced/ transformed strains, which may contain some new genes.	Not considered relevant		
	Protoplast Fusion & Selection	Microorganisms without exogenous DNA and with a genome sequence that is almost identical to the parental line - with the exception of some limited targeted changes - **in contrast** to protoplast fusion strains, which may contain many (even hundreds or thousands) of new genes.			Plants with a new allele (gene variant) inserted in a targeted way at a precise position in the genome **in contrast** to protoplast fusion strains, which may contain many (even hundreds or thousands) of new genes.	Not considered relevant		

*Genome Editing: includes amongst others ZFNs, TALEN, CRISPR/Cas and meganucleases

Table 5A - NBT compared with CBT – Ease of Use /Efficiency

	NBT / CBT	Genome editing*				Techniques introducing genetic material from same or sexually compatible species		Techniques in which transgenes are introduced in an intermediate step
		ODM	**SDN1**	**SDN2**	**SDN3**	**Cisgenesis**	**Intragenesis**	**Reverse Breeding**
Plants	**Mutation Breeding**	The technique is simpler than SDN1 and 2, but has a low efficiency. Compared with mutation breeding which required screening of several thousand mutants, genome editing is more efficient through the use of simplified screening (can save 6 to 8 years to get the commercial variety depending on the species, the targeted genomic locus, the new desired trait and on the possibility to select for desired mutations on the basis of the phenotype). Since most genome editing applications require an in vitro phase, ease of use depends on how amenable the target plants are for in vitro culture. ODM allows the directed modification of several nucleotides which is impossible through mutation breeding. It can be replaced by SDN2 which is much more efficient.	Among the different genome editing systems, the ones related to CRISPR are the easiest to handle and the most efficient. Compared with mutation breeding which required screening of several thousand mutants, genome editing is more efficient through the use of simplified screening (can save 6 to 8 years to get the commercial variety depending on the species, the targeted genomic locus, the new desired trait and on the possibility to select for desired mutations on the basis of the phenotype). Since most genome editing applications require an in vitro phase, ease of use depends on how amenable the target plants are for in vitro culture. SDN1 is already applied to a large extent.	Among the different genome editing systems, the ones related to CRISPR are the easiest to handle and the most efficient. Compared with mutation breeding which required screening of several thousand mutants, genome editing is more efficient through the use of simplified screening (can save 6 to 8 years to get the commercial variety depending on the species, the targeted genomic locus, the new desired trait and on the possibility to select for desired mutations on the basis of the phenotype). Since most genome editing applications require an in vitro phase, ease of use depends on how amenable the target plants are for in vitro culture. Homologous recombination is however less frequent than non-homologous end-joining and the procedure will therefore generate many more „SDN1-like“ products than the required „SDN2“ product. The approach is more complex and laborious than SDN1 but might replace ODM because of its higher efficiency.	Not considered relevant			
	Crossing	See ODM vs. Mutation Breeding in Plants (above)	See SDN1 vs. Mutation Breeding in Plants (above)	See SDN2 vs. Mutation Breeding in Plants (above)	There are insufficient publically accessible data to enable comparison.	Much more efficient than introduction of the same alleles by sexual crossing (if at all possible) due to limited need for backcrossing because the desired allele is introduced without any linkage drag of other unwanted alleles.	The products obtained by intragenesis could not be obtained by sexual crossing.	Parental lines cannot be obtained back from the progeny of a conventional cross. Reverse breeding offers to do just that. Until now, however, only for the model plant Arabidopsis thaliana published data are available.
Animals	**Breeding**	The technique is simpler but has a low efficiency. The efficiency depends on the species; the targeted genomic locus and whether selection is possible based on the phenotype or requires detection by molecular analysis. It can be replaced by SDN2 which is much more efficient.	Among the different genome editing systems, the ones related to CRISPR are the easiest to handle and the most efficient. The SDN1 method is simpler and is already applied to a large extent.	Among the different genome editing systems, the ones related to CRISPR are the easiest to handle and the most efficient. Homologous recombination is however less frequent than non-homologous end-joining and the procedure will therefore generate many more „SDN1-like“ products than the required „SDN2“ product. The approach is more complex and laborious than SDN1 but might replace ODM because of its higher efficiency.	There are insufficient publically accessible data to enable comparison.	Not considered relevant		
Microorganisms	**Induced Mutagenesis & Selection**	Genome editing techniques are simpler and can be applied for *gene knock out* or for introducing variations, including naturally existing ones. Genome editing efficiency depends on the microbial species and whether selection or phenotypic screening is possible for the trait or requires detection by molecular analysis.			Not considered relevant	Not considered relevant		
	Conjugation/ Transduction or Transformation & Selection				The technique can be easier, in particular for the introduction of multiple DNA fragments as they are inserted precisely at the chosen position(s) in the genome.			
	Protoplast Fusion & Selection							

*Genome Editing: includes amongst others ZFNs, TALEN, CRISPR/Cas and meganucleases

Table 6A - NBT compared with CBT - Speed-Cost

	NBT / CBT	Genome editing*				Techniques introducing genetic material from same or sexually compatible species		Techniques in which transgenes are introduced in an intermediate step
		ODM	**SDN1**	**SDN2**	**SDN3**	**Cisgenesis**	**Intragenesis**	**Reverse Breeding**
Plants	**Mutation Breeding**	Faster, cheaper and more efficient techniques (can save 6 to 8 years to get the commercial variety depending on the species, the new desired trait and on the possibility to select for desired mutations on the basis of the phenotype) mainly due to the fact that mutation breeding requires a higher number of generations of repeated backcrosses to eliminate unintended mutations.			Not considered relevant			
	Crossing	Faster, cheaper and more efficient technique (savings 6 to 8 years to get the commercial variety depending on the species, the new desired trait and on the possibility to select for desired mutations on the basis of the phenotype) mainly due to the fact that sexual crosses require a higher number of generations of repeated backcrosses to eliminate undesired alleles.	Faster, cheaper and more efficient technique because selection of mutants downstream is greatly facilitated (savings 6 to 8 years to get the commercial variety depending on the species, the new desired trait and on the possibility to select for desired mutations on the basis of the phenotype). Multiple targeted mutations can also be introduced at the same time.	Faster and cheaper due to targeted introduction of mutations (time savings 6 to 8 years to get the commercial variety depending on the species, the new desired trait and on the possibility to select for desired mutations on the basis of the phenotype). Due to the lower frequency of modification SDN2 is not as efficient as SDN1 and subsequently the screening for the intended modification can be longer and more difficult, but still less time consuming than backcrossing.	Much faster due to limited need for backcrossing because of targeted insertion of DNA fragment at precise location, therefore less costly.	Much faster (time savings of up to 50 years in the case of scab-resistant apple) and therefore less costly due to limited need for backcrossing because the desired allele is introduced without any linkage drag of other unwanted alleles, especially important for time saving in case of an allele obtained from a wild relative.		Parental lines cannot be obtained back from the progeny of a conventional cross. Reverse breeding offers to do just that. Until now, however, only for the model plant Arabidopsis thaliana published data are available.
Animals[1]	**Breeding**	Faster and cheaper due to increased efficiency leading to the targeted introduction of mutations which reduces the complex selection screen based on the phenotype. The speed of genome editing success is critically dependent upon the genetic trait (rapid in monogenic traits and possibly much slower in more complex regulated traits). As for all breeding techniques speed is also largely dependent on the generation interval that is very different between farm animal species.	**As for ODM vs Conventional Breeding (see left).** With the help of SDN1 multiple controlled mutations can be also introduced at the same time leading to further time and cost savings.	**As for ODM vs Conventional Breeding (see left).** Due to the lower frequency of modification SDN2 is not as efficient as SDN1 and subsequently the screening for the intended modification can be longer and more difficult.	Much faster and less costly due to limited need for screening because of targeted insertion of DNA fragment at precise location.	Not considered relevant		
Microorganisms	**Induced Mutagenesis & Selection**	Selection and screening of ODM, SDN1 and SDN2 mutants can be much less costly and faster than for randomly induced mutations, with the exact time and cost savings depending on the desired trait.			Not considered relevant			
	Conjugation/ Transduction or Transformation & Selection	Selection and screening of ODM, SDN1, SDN2 or SDN3 mutants can be much less costly and faster than for conjugation, with the exact time and cost savings depending on the desired trait.				Not considered relevant		
	Protoplast Fusion & Selection	Selection and screening of ODM, SDN1, SDN2 or SDN3 mutants can be much less costly and faster than for protoplast fusion, with the exact time and cost savings depending on the desired trait.				Not considered relevant		

*Genome Editing: includes amongst others ZFNs, TALEN, CRISPR/Cas and meganucleases

Table 7A - NBT compared with CBT - Maturity

	NBT / CBT	Genome editing*				Techniques introducing genetic material from same or sexually compatible species		Techniques in which transgenes are introduced in an intermediate step
		ODM	**SDN1**	**SDN2**	**SDN3**	**Cisgenesis**	**Intragenesis**	**Reverse Breeding**
Plants	**Mutation Breeding** is used since the 1920s. Today, more than 3200 crop varieties obtained with mutation breeding are available	Proofs of concept have been obtained on maize, tobacco, rice, flax, and potato. CIBUS has commercialized in Canada an herbicide resistant rapeseed variety in 2017.	Proofs of concept have been obtained for example for wheat and rice. For some SDN1 products authorisation has been requested, others are ready for field trials. Field trials of waxy (starch composed of only amylopectin) corn hybrids and corn with improved drought tolerance are being performed by Pioneer USA.	Several products have been obtained as a proof of concept, *e.g.* cucumber resistant to potyvirus, but technique needs further improvement for efficiency before broad application. Cellectis intends to commercialize in 2018 an edited soybean modified for oil composition.	Not considered relevant			
	Crossing is the most widely used plant breeding technique, systematically used since the 19th century (discovery of Mendelian laws)				Laboratory experiments have shown the feasibility in model plants, but there are insufficient publically accessible data for making a substantiated statement on maturity.	Products are close to reach the market: *e.g.* the *Phytophthora*-resistant potato and scab-resistant apple which are in field trials.	Laboratory experiments have shown the feasibility in model plants, but there are insufficient publically accessible data for making a substantiated statement on maturity.	Concept developed in the model plant *Arabidopsis thaliana*.
Animals	**Breeding** Scientifically based animal breeding strategies making use of biotechnology have existed for about 60 years.	Gene editing is an important new tool in livestock breeding *(e.g.* PRRS resistant pigs and tuberculosis resistant cattle) with the potential of broader application depending on increased knowledge of the farm animals' genome. It is specifically useful for species (cattle, pigs, sheep, etc.) in which homologous recombination techniques cannot be applied due to the lack of true germ line competent embryonic stem cells.				Not considered relevant		
Microorganisms	**Conventional techniques** in use for more than 50 years	Proofs of concept have been obtained in bacteria and yeast.				Not considered relevant		

*Genome Editing: includes amongst others ZFNs, TALEN, CRISPR/Cas and meganucleases

4.3. New techniques (NBT) and established techniques of genetic modification (ETGM)

Tables 1B to 7B compare the NBT and ETGM, for plants, animals and microorganisms and for the different criteria of the scoping paper.

The statements made in the tables are made from the point of view of the NBT, mentioned on the X-axis, in the light of the ETGM mentioned on the Y-axis. Where applicable, comparative statements are made and where possible in a quantitative way, otherwise in a qualitative way. The tables also contain some general statements which apply to all breeding techniques. The reader should refer to the main body of the text for references supporting the statements made in the tables.

For farm animals the NBT of cisgenesis, intragenesis, agro-infiltration, reverse breeding and RdDM are not relevant for comparison as they are currently not applied. Likewise for microorganisms, agroinfiltration, reverse breeding and RdDM are not relevant for comparison as they are not applied.

In microorganisms, a process analogous to cisgenesis and intragenesis is self-cloning. It is defined as the re-introduction of a host's own DNA which may have been altered or the introduction of DNA from a closely related strain of the same or a closely related species.

Under Directive (EC) 98/81 the term self-cloning means the removal of nucleic acid from an organism and the reinsertion of all or part of that nucleic acid into the same organism, with or without prior enzymic or mechanical steps. It is assumed that no nucleic acid from another organism is introduced, and that neither the organism nor the nucleic acid is recombinant.

In the case of plants the comparison of NBT: ODM, SDN1 and SDN2, agro-infiltration, Reverse breeding and RdDM are not relevant as they introduce changes (point mutations) which are not comparable to the changes introduced by ETGM (introduction of DNA fragments).

Agro-infiltration, reverse breeding and RdDM in plants introduce transgenes only as an intermediary step. They do not aim to construct transgenic

plants, but may use the transgene-free offspring of transgenic lines (reverse breeding, RdDM) or local transformation (agro-infiltration) in part of the plants to obtain a systemic (epigenetic) effect in the whole plant (for instance pathogen resistance) by the spread of small RNA molecules to promote gene silencing. Recent advances suggest that instead of generating intermediate transgenic plants chemically synthesized sRNA molecules may be sprayed on plants to induce gene silencing in the plant itself or in a pathogen infecting the plant.

In the case of ETGM in plants and animals one or several transgenes are inserted into the genome in an uncontrolled process at random positions. The genes may be inserted in one or multiple copies which may be present at one locus in the genome or at multiple loci. For obtaining the end products organisms are selected which contain only one copy of the transgene. In the case of plants additional copies of the transgene can be eliminated through backcrossing. NBT seek to control the process of exogenous DNA molecule integration to target the new genes to a specific location in the genome using the SDN3 procedure so that undesired mutations are avoided and undesired effects on the expression of non-targeted genes or the gene itself (position effects) are minimized. ETGM of bacteria and yeast already uses HR to target transgenes to a specific site in the genome. Employment of SDN3 increases the efficiency of this process.

4.3.1. Detectability/Identification

Analytical methods (*e.g.* PCR or DNA sequencing) for the detection of genetic changes in plants, animals and microorganisms are available, but require prior knowledge. This is in particular necessary to identify the products of intragenesis and cisgenesis in plants, as similar or almost identical genes are already present in the genome.

Plant end products lacking gene insertions obtained from procedures involving agro-inoculation, RdDM and reverse breeding are indistinguishable at the DNA level from natural crop lines.

In plants, animals and microorganisms, without any prior information and where the introduced changes cannot be postulated, *e.g.* from information about existing registered GMOs in databases, detection becomes more challenging and the differentiation from naturally occurring events and the identification of the underlying technique is generally impossible.

In microorganisms the detection of changes by WGS without prior knowledge is easier as compared with plants and animals because of the smaller and less complex genome.

4.3.2. Unintended effects

For all animals and plants, unintended effects of ETGM include position effects, which affect the expression of the transgene itself and/ or of the neighbouring genes due to the insertion of the transgene or interference with their expression. These uncertainties are strongly reduced when using SDN3 for insertion, as a specific integration site chosen. This enables stable expression of the transgene and minimises undesired effects on the phenotype due to effects on neighbouring genes. When a new gene is introduced by SDN3 or ETGM it can interact with the whole set of endogenous genes of the recipient organism. Potential desired and undesired effects of this cannot always be predicted. Therefore products must be tested for performance, *e.g.* for plants in field experiments.

The ETGM of microorganisms usually already involves insertion of transgenes via homologous recombination. SDN3 increases the efficiency of this process, and the end product can be identical to that of ETGM.

4.3.3. Presence of exogenous DNA

In SDN3 genome editing for plants, exogenous DNA in the form of a gene linked to the desired trait is present in the end product at a precisely defined insertion site, in contrast to plants and animals obtained with ETGM, which contain exogenous DNA at random positions in the genome.

In NBT which introduce genetic material from the same or a sexually compatible species, an exogenous DNA molecule in the form of one or a few cisgenes or intragenes is present in the end product. Contrastingly, plants

obtained with ETGM also contain exogenous DNA, but usually in the form of transgenes (from sexually incompatible species).

For microorganisms obtained by SDN3, exogenous DNA in the form a gene linked to the desired trait is intentionally inserted at a specified site into the end product – comparable to end products obtained with ETGM.

4.3.4. End-product

The plant end-product obtained by employing the genome editing technique SDN3 is a plant with a new gene (cisgene, intragene or transgene) inserted in a targeted way at a defined position in the genome, in contrast to plants obtained with ETGM containing exogenous DNA, usually a transgene, at a random location. The plant end-product of cisgenesis or intragenesis is a plant with genetic material from the same or a sexually compatible species while plants obtained with ETGM usually contain a transgenic DNA molecule (from a sexually incompatible species).

In the case of animals, the end product obtained with the application of SDN3 is an animal with an exogenous DNA fragment (usually a transgene) inserted in a targeted way at a defined position in the genome in contrast to animals obtained with ETGM introducing a transgenic DNA molecule at a random genome location.

The microorganism end-product of the NBT of gene editing by SDN3 is a microorganism with an exogenous DNA fragment (from the same, a sexually compatible species, or a sexually incompatible species) inserted in a targeted way at a precise position in the genome, comparable to a microorganism obtained with ETGM.

The animal end-product of an NBT of gene editing by SDN3 is an animal with exogenous DNA, usually a transgene, inserted in a targeted way at a precise position in the genome in contrast to an animal obtained with ETGM contain the transgene at a random position in the genome.

4.3.5. Ease of Use and Efficiency

In the case of animals and plants the induction of targeted genetic modifications in livestock species or crop species was virtually not possible with the ETGM. This has changed with the emergence of SDNs.

When compared to the ETGM, targeted integration of transgenes (or cisgenes) can now be obtained with much higher efficiency by the aid of SDNs which can cut the genome at the integration site. Integration can then occur with much higher frequency by either NHEJ (constructs lacking homology with the genome) or preferentially by HR. The latter offers better control of transgenesis and cisgenesis/ intragenesis as it avoids copy number and position effects.

ETGM in farm animals: such as microinjection of exogenous DNA into pronuclei of zygotes; the use of transfected donor cells in SCNT; or the use of lentiviral vectors is usually hampered by low efficiency, random integration patterns and multiple insertion events and is not compatible with targeted genetic modifications that are a major prerequisite for the production of well characterised animals and products thereof.

Compared with ETGM, DNA nucleases can increase the targeting rate 10,000-fold. A significant increase to 1-18% homologous recombination events per mammalian cell was achieved when the targeted double-strand break was introduced by SDNs compared with 10^{-6} HR (homologous recombination) events without the use of SDNs. This renders HR for the first time routinely applicable in farm animals.

In microorganisms, genome editing can facilitate and accelerate the targeted introduction of mutations, in particular the introduction of multiple alterations in the genome, as demonstrated for non-food applications (Cobb, Wang, & Zhao, 2014). Thus, Huang et al (2015) (Huang, Zheng, Jiang, Hu, & Lu, 2015) describe the successful deletion of whole antibiotic synthesis clusters (21-83 kb) in *Streptomyces*. In *Escherichia coli* and *Tatumella citrea* several genes could be inserted or deleted simultaneously

with CRISPR based methods with high efficiency which is impossible with other techniques (Y. Jiang et al., 2015).

4.3.6. Speed/Cost

For crop plants as well as for animals it has been virtually impossible to obtain targeted integrations with CBT or ETGM. With SDN3 this is now becoming a reality, which can lower the cost to the market significantly because of the targeted integration at a specific site chosen to enable stable expression of the transgene and to minimise undesired effects on the phenotype due to effects on neighbouring genes. Therefore it is not necessary to characterise and compare all the transgenic lines for performance, but it is sufficient to pick a few for future use and commercialisation.

As for all breeding techniques in animals the speed of genome editing techniques is also largely dependent on the generation interval that differs a lot between farm animal species.

For microorganisms the speed and frequency at which genes are introduced using SDN3 is often higher compared with ETGM, which means that new changes can be made in terms of days rather than weeks which is likely to lower the costs for designing new microorganisms for food and feed applications.

4.3.7. Maturity

For plants proof of concept of SDN3 has been obtained in model plants using constructs immediately in a transformation procedure or after first integrating into the genome at a random position but then in the next generation used as a substrate for gene targeting by SDN3 ('*in* planta gene targeting').

Crops and horticulture plants modified with cisgenesis are close to reaching the market, *e.g.* the *Phytophthora*-resistant potato and scab-resistant apple (containing the vf gene of *Malus floribunda*), which are in field trials.

Practical applications of the genome editing techniques in animals already exist for biomedical applications, in particular for the production of pharmaceutical proteins in milk (gene pharming: GTC Biotherapeutics, Pharming, Biosidus) and xenotransplantation, *i.e.* the production of porcine organs and tissue in human organ transplantation. Agricultural applications are now also rapidly emerging (see 3.3.1, *e.g.* PRRS resistant pigs and tuberculosis resistant cattle).

For microorganisms proofs of concept for SDN3 have been obtained on bacteria, yeast and fungi. Microorganisms edited with the CRISPR-Cas system are already commercially used for food and feed applications, *e.g.* in starter cultures for dairy fermentations (yogurt and cheese).

List of Tables

NBT compared with ETGM

Table 1B - NBT compared with ETGM - Detectability/Identification

	NBT / ETGM	Genome editing*				Techniques introducing genetic material from same or sexually compatible species		Techniques in which transgenes are introduced in an intermediate step
		ODM	SDN1	SDN2	SDN3	Cisgenesis	Intragenesis	Reverse Breeding
Plants		Not considered relevant			Detection of changes is possible with a variety of analytical methods, if prior information on the introduced fragment(s) is available, i.e. if there is a known target to search for (for products of ETGM this information is provided together with the request for authorisation). If there is no information regarding the introduced fragment(s) (i.e. no known target for which to search), detection of changes is more challenging. It can be possible with whole genome sequencing (WGS), and in combination with other approaches, but only with a suitable reference genome for comparison. The likelihood that changes are not detected is smaller than for ODM, SDN1 and SDN2, as larger changes are introduced by SDN3, cisgenesis and intragenesis (insertion of DNA fragments). Identification of the technique underlying the detected changes and distinction from natural variation solely with analytical methods is not possible. For instance, in the case of SDN3 the same changes can be introduced by EGTM.			Not considered relevant
Animals		Not considered relevant			Detection of changes is possible with a variety of analytical methods, if prior information on the introduced fragment(s) is available, i.e. if there is a known target to search for (for products of ETGM this information is provided together with the request for authorisation). If there is no information regarding the introduced fragment(s) (i.e. no known target for which to search), detection of changes is more challenging. It can be possible with WGS, and in combination with other approaches, but only with a suitable reference genome for comparison. Identification of the technique underlying the detected changes and distinction from natural variation solely with analytical methods is not possible. Depending on the introduced fragment the same change can be introduced by ETGM.	Not considered relevant		
Microorganisms		Detection of changes is possible with a variety of analytical methods, if prior information on the introduced changes is available, i.e. if there is a known target to search for (for products of established GM techniques this information is provided together with the request for authorisation). If there is no information regarding the introduced changes (i.e. no known target for which to search), detection of changes is more challenging. It can be possible with WGS, and in combination with other approaches, but only with a suitable reference genome for comparison. Identification of the technique underlying the detected changes and distinction from natural variation solely with analytical methods is not possible. For instance, in the case of SDN3 the same fragment can be introduced by ETGM or by a natural process of horizontal gene transfer.				Not considered relevant		

*Genome Editing: includes amongst others ZFNs, TALEN, CRISPR/Cas and meganucleases

Table 2B - NBT compared with ETGM – Unintended Effects

	NBT / ETGM	Genome editing*				Techniques introducing genetic material from same or sexually compatible species	
		ODM	**SDN1**	**SDN2**	**SDN3**	**Cisgenesis**	**Intragenesis**
General		Oligonucleotides used for inducing mutations are usually degraded and not integrated into the genome. However, the possibility of their integration into the genome (as an unintended effect) cannot be completely excluded. The presence of unintended exogenous DNA integrated into the genome can be detected with appropriate analytical methods (*e.g.* PCR, WGS, for technical restrictions see section 4.1). If exogenous DNA is detected, the organism will not be used as the end product.	In the case of the introduction of transgene sequences providing stable or transient expression of SDN proteins and guide RNAs, these DNA sequences may be integrated into the genome. The presence of unintended exogenous DNA integrated into the genome can be detected with appropriate analytical methods (*e.g.* PCR, WGS, for technical restrictions see section 4.1). If exogenous DNA is detected, the organism will not be used as the end product.	In the case of the introduction of transgene sequences providing stable or transient expression of SDN proteins and guide RNAs, these DNA sequences may be integrated into the genome (like in SDN1). Oligonucleotides used for inducing mutations are usually degraded and not integrated into the genome. However, the possibility of their integration into the genome (as an unintended effect) cannot be completely excluded (like in ODM). The presence of unintended exogenous DNA integrated into the genome can be detected with appropriate analytical methods (*e.g.* PCR, WGS, for technical restrictions see section 4.1). If exogenous DNA is detected, the organism will not be used as the end product.	In the case of the introduction of transgene sequences providing stable or transient expression of SDN proteins and guide RNAs, these DNA sequences may be integrated into the genome. The presence of unintended exogenous DNA integrated into the genome can be detected with appropriate analytical methods (*e.g.* PCR, WGS, for technical restrictions see section 4.1). If exogenous DNA is detected, the organism will not be used as the end product.	Not considered relevant	
Plants		Not considered relevant			**Lower** frequency because insertion is targeted which minimises unintended effects associated with the disruption of genes and/or regulatory elements in the recipient genome as well as position effects (expression level of inserted gene depending on precise location). In cases of unintended integration of exogenous DNA, it can be removed within one generation by backcrossing.	**Same or potentially lower**, in particular considering the probability of pleiotropic effects, because only new alleles from a sexually compatible species are introduced.	**Comparable to** ETGM
Animals		Not considered relevant			**Lower** frequency or complete lack of unintended effects because mutations (substitution/ deletion/ insertion) are targeted and not random, which minimises unintended effects associated with the disruption of genes and/or regulatory elements in the recipient genome. As for ETGM, potential unintended pleiotropic or effects of the genetic background cannot be completely ruled out, but are very unlikely based on current knowledge of gene functions.	Not considered relevant	
Microorganisms		**Comparable frequency** as with ETGM. ODM, SDN1, and SDN2 can generate mutations at precise positions, and for microbial genomes it is also possible with ETGM.			**Comparable frequency** as with ETGM. SDN3 can insert DNA sequences at precise positions, and for the microbial genome it is also possible with ETGM.	Not considered relevant	

*Genome Editing: includes amongst others ZFNs, TALEN, CRISPR/Cas and meganucleases

Table 3B - NBT compared with ETGM – Presence of Exogenous DNA molecule

	NBT / ETGM	Genome editing*				Techniques introducing genetic material from same or sexually compatible species	
		ODM	SDN1	SDN2	SDN3	Cisgenesis	Intragenesis
Plants		Not considered relevant			Exogenous DNA** in the form of a gene linked to the desired trait is present in the end product at a precisely defined insertion site in contrast to plants obtained with ETGM, which contain exogenous DNA at random positions in the genome. For potential presence of exogenous DNA as an unintended effect in an intermediate product - see the general remark in Table 2A. In cases of unintended integration of exogenous DNA, it can be removed within one generation by backcrossing.	Exogenous DNA in the form of one or a few cisgenes (allele(s) from the same or a sexually compatible species) is present in the end product in contrast to plants obtained with ETGM, which also contain exogenous DNA, but usually in the form of transgenes (from sexually incompatible species).	Exogenous DNA in the form of one or a few intragenes (artificially constructed alleles with sequences from the same or a sexually compatible species) is present in contrast to plants obtained with ETGM, which also contain exogenous DNA, but usually in the form of transgenes (from sexually incompatible species).
Animals		Not considered relevant			Exogenous DNA in the form of a transgene is present in the end product at a precisely defined insertion site in contrast to animals obtained with ETGM, which contain the transgene at random positions in the genome. For potential presence of exogenous DNA as an unintended effect in an intermediate product - see the general remark in Table 2A.	Not considered relevant	
Microorganisms		No exogenous DNA present in the end product. For potential presence of exogenous DNA as an unintended effect in an intermediate product - see the general remark in Table 2A. End products with point mutations introduced by ETGM through homologous recombination contain exogenous DNA in the form of a fragment from the same or sexually compatible species.			Exogenous DNA in the form of a gene linked to the desired trait is present in the end product at a precisely defined insertion site. In microorganisms this is, in contrast to the situation in plants and animals, comparable to end products obtained with ETGM. For potential presence of exogenous DNA as an unintended effect in an intermediate product - see the general remark in Table 2A.	Not considered relevant	

*Genome Editing: includes amongst others ZFNs, TALEN, CRISPR/Cas and meganucleases

**For the purpose of this explanatory note the term exogenous DNA is defined as DNA originating outside the organism of concern or under investigation which can be introduced naturally or by technological intervention

Table 4B - NBT compared with ETGM– End-products

	NBT / ETGM	Genome editing*				Techniques introducing genetic material from same or sexually compatible species	
		ODM	SDN1	SDN2	SDN3	Cisgenesis	Intragenesis
Plants		Not considered relevant			Plants with a new gene inserted in a targeted way at a chosen position in the genome **in contrast** to plants obtained with ETGM, in which transgenes are inserted in a non-targeted way at random positions in the genome.	Plants with one or a few new alleles from the same or a sexually compatible species (cisgene) **in contrast** to plants obtained with ETGM, which usually contain a transgene from a sexually incompatible species.	Plants with one or a few new artificially constructed alleles with sequences from the same or a sexually compatible species **in contrast** to plants obtained with ETGM, which usually contain a transgene from a sexually incompatible species
Animals		Not considered relevant			Animals with a new gene inserted in a targeted way at a chosen position in the genome **in contrast** to animals obtained with ETGM which contain a transgene at a random position in the genome.	Not considered relevant	
Microorganisms		Microorganisms with point mutations and without exogenous DNA. In contrast, microorganisms with point mutations introduced by ETGM through homologous recombination contain exogenous DNA in the form of a fragment from the same or sexually compatible species.			Microorganisms with a new gene inserted in a targeted way at a chosen position in the genome, **comparable** to microorganisms obtained with ETGM.	Not considered relevant	

*Genome Editing: includes amongst others ZFNs, TALEN, CRISPR/Cas and meganucleases

Table 5B - NBT compared with ETGM – Ease of Use /Efficiency

	NBT / ETGM	Genome editing*				Techniques introducing genetic material from same or sexually compatible species	
		ODM	SDN1	SDN2	SDN3	Cisgenesis	Intragenesis
Plants		Not considered relevant			Generally simpler and more straightforward as SDN3 enables integration of the desired DNA fragments at the chosen positions in the genome. Efficiency mainly depends on the targeted genome locus. Can facilitate insertion of novel genes and is used in combination with ETGM. Induction of multiple genetic modifications is possible. Amongst the genome editing techniques, the CRISPR system is currently the most often employed owing to versatility and easiness to use.	Ease of use and efficiency are similar to transgenesis (ETGM).	
Animals		Not considered relevant			Generally simpler and more straightforward as SDN3 enables integration of the desired DNA fragments at the chosen positions in the genome. Efficiency mainly depends on the targeted genome locus. Can facilitate insertion of novel genes and is used in combination with ETGM. Induction of multiple genetic modifications is possible. Amongst the genome editing techniques, the CRISPR system is currently the most often employed owing to versatility and easiness to use.	Not considered relevant	
Microorganisms		Genome editing techniques can be simpler and can be applied for *gene knock out* or for introducing variations, including naturally existing ones. Genome editing efficiency depends on the microbial species and whether selection or phenotypic screening is possible for the trait or requires detection by molecular analysis. In many cases the new techniques can represent an improvement of ETGM by easing the targeted insertion of multiple genetic modifications in a microorganism.			Comparable to ETGM for the introduction of a single DNA fragment. For the introduction of multiple fragments the application of SDN3 can be easier and more efficient as compared to ETGM.	Not considered relevant	

Table 6B - NBT compared with ETGM - Speed-Cost

	NBT / ETGM	Genome editing*				Techniques introducing genetic material from same or sexually compatible species	
		ODM	SDN1	SDN2	SDN3	Cisgenesis	Intragenesis
Plants		Not considered relevant			Faster and cheaper due to increased efficiency and targeted introduction of DNA fragments at the desired genome location, which reduces the complex selection screen. The speed of genome editing success is critically dependent upon the genetic trait (rapid in monogenic traits and possibly much slower in more complex regulated traits).	Speed and costs similar to transgenesis (ETGM)	
Animals		Not considered relevant			Faster and cheaper due to increased efficiency and targeted introduction of DNA fragments at the desired genome location which reduces the complex selection screen. The speed of genome editing success is critically dependent upon the genetic trait (rapid in monogenic traits and possibly much slower in more complex regulated traits).	Not considered relevant	
Microorganisms		Selection of ODM, SDN1, SDN2 and SDN3 mutants can be faster and thus cheaper than for ETGM.				Not considered relevant	

*Genome Editing: includes amongst others ZFNs, TALEN, CRISPR/Cas and meganucleases

Table 7B - NBT compared with ETGM – Maturity

	NBT / ETGM	Genome editing*				Techniques introducing genetic material from same or sexually compatible species	
		ODM	SDN1	SDN2	SDN3	Cisgenesis	Intragenesis
Plants	Transgenic field crops generated with ETGM are commercialised since 1996.	Not considered relevant			Laboratory experiments have shown the feasibility in model plants, but there are insufficient publically accessible data for making a substantiated statement on maturity.	Products are close to reach the market: *e.g.* the *Phytophthora*-resistant potato and scab-resistant apple which are in field trials.	Laboratory experiments have shown the feasibility in model plants, but there are insufficient publically accessible data for making a substantiated statement on maturity.
Animals	First transgenic livestock was reported in 1985.	Gene editing is an important new tool in livestock breeding (*e.g.* PRRS resistant pigs and tuberculosis resistant cattle), with the potential of broader application depending on increased knowledge of the farm animals' genome. It is specifically useful for species (cattle, pigs, sheep, etc.) in which homologous recombination techniques cannot be applied due to the lack of true germ line competent embryonic stem cells.				Not considered relevant	
Microorganisms	The first microorganisms generated with ETGM for food/feed production (microbial enzymes) were authorised in the USA in the late 1980s.	Proofs of concept have been obtained in bacteria and yeast.				Not considered relevant	

*Genome Editing: includes amongst others ZFNs, TALEN, CRISPR/Cas and meganucleases

Annexes

ANNEXES

Annex 1 – Scoping Paper

Scoping paper:
New techniques in agricultural biotechnology

25 November, 2016

New techniques in agricultural biotechnology

Policy context

During the past decade a number of new techniques have been developed leading to organisms in which genetic material is altered compared to the initial organism. These techniques are attracting interest for their use in agriculture for plant and animal breeding. Many of these can also be used for microbial applications. A wide debate amongst stakeholders and the general public is ongoing within and outside the EU concerning the use in agriculture of organisms produced with these techniques, in particular relating to their comparison with 1) conventional breeding techniques and 2) established techniques of genetic modification. The use of some of these techniques in the field of synthetic biology and for gene drive can also be relevant for agricultural applications.

For the purpose of this scoping paper the terms:

- "established techniques of genetic modification in biotechnology" refers to various genetic engineering techniques which have been significantly used over the last 30 years to produce genetically modified organisms[1];
- "conventional breeding techniques" refers to traditionally used techniques[2]
- "new techniques" refers to techniques used in biotechnology other than those covered by the terms above. The term includes but is not limited to:

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[1] Directive 2001/18/EC of the European Parliament and of the Council of 12 March 2001 on the deliberate release into the environment of genetically modified organisms and repealing Council Directive 90/220/EEC , OJ L 106, 17.4.2001, p. 1–39.

Regulation (EC) No 1829/2003 of the European Parliament and of the Council of 22 September 2003 on genetically modified food and feed, OJ L 268, 18.10.2003, p. 1–23.

[2] A wide range of techniques is used in conventional breeding. In its *Scientific Opinion addressing the safety assessment of plants developed using Zinc Finger Nuclease 3 and other Site-Directed Nucleases with similar function* (EFSA Journal 2012;10(10):2943), EFSA lists the following conventional plant breeding techniques as relevant for a comparison with plants developed by the SDN-3 technique: sexual crosses, bridge crosses, embryo rescue, somatic hybridisation, translocation breeding and mutation breeding. In animal breeding, in addition to natural mating, assisted reproductive techniques have contributed to genetic selection during past decades. In its *Scientific Opinion of the Scientific Committee on a request from the European Commission on Food Safety, Animal Health and Welfare and Environmental Impact of Animals derived from Cloning by Somatic Cell Nucleus Transfer (SCNT) and their Offspring and Products Obtained from those Animals* (EFSA Journal (2008) 767, 1-49) EFSA mentions the following technologies: artificial insemination from selected sires with its possible extension to sexed semen, oocyte collection from selected dams, embryo selection and transfer from selected genitors, *in vitro* fertilisation, and the long term storage of gametes and embryos.

- the techniques identified in the 2011 Member States' Expert Group report Oligonucleotide Directed Mutagenesis (ODM); Zinc Finger Nuclease (ZFN) Technology (ZFN-1, -2, -3); Cisgenesis and Intragenesis; Agro-infiltration ("*senso stricto*" and "floral dip"); RNA-dependent DNA methylation (RdDM) and Reverse Breeding.
    - more recent genome editing technologies, such as Transcription activator-like effector nucleases (TALEN), meganucleases and Clustered regularly interspaced short palindromic repeats (CRISPR).
- "synthetic biology" means the application of science, technology and engineering to facilitate and accelerate the design, manufacture and/or modification of genetic materials in living organisms[3].
- "gene drive" means stimulating biased inheritance of particular genes to alter entire populations.

Scientific advice previously requested by the Commission

The Commission has in the past obtained scientific advice on new breeding techniques.

The study on "New Plant Breeding Techniques: state-of-the-art and prospects for commercial development" carried out by the Commission's Joint Research Centre (JRC). The study (report published in 2011[4]) investigates the degree of development and adoption by the commercial breeding sector of new plant breeding techniques, discusses drivers and constraints for further developments and evaluates the technical possibilities for detecting and identifying crops produced by new plant breeding techniques.

A Member States' Expert Group established a list of new plant breeding techniques and evaluated them in the light of the existing legislation and of the most recent available scientific data. The Expert Group also addressed synthetic biology applications, but provided a very limited analysis on this topic. The group finalised its report in December 2011[5], but could not reach a consensus on all techniques.

The EFSA Panel on GMOs adopted scientific opinions on three techniques, namely cisgenesis, intragenesis and site directed nucleases technique, in terms of the risks they might pose and the applicability of the existing EFSA guidance documents on GM plants for their risk assessment. EFSA's opinions have been published in 2012[6].

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3 SCENIHR, SCCS, SCHER (2014) Synthetic Biology I Definition, Opinion, September 2014. Available from: http://ec.europa.eu/health/scientific_committees/emerging/docs/scenihr_o_044.pdf

4 http://ipts.jrc.ec.europa.eu/publications/pub.cfm?id=4100

5 New Techniques Working Group FINAL REPORT

6 EFSA Journal 2012;10(2):2561, EFSA Journal 2012;10(10):2943

The three Scientific Committees SCHER, SCENIHR and SCCS, upon request from the Commission, published three opinions on synthetic biology, focusing on its scope and definition, risk assessment methodologies and safety aspects and research priorities[7].

This previous advice is focussed on new techniques for plant breeding as well as on synthetic biology and provides a good basis and relevant information in relation to these subjects. However, considering the rapid and recent developments in the field, and the broader scope of the possible applications of the new techniques in agricultural biotechnology, which also concern the application of these techniques to animals and micro-organisms, an up-to-date explanatory note/opinion and scientific advice are requested to the SAM HLG as described below.

Request to SAM HLG

SAM HLG is asked in the first instance and by March 2017 to provide an explanatory note on *new techniques in agricultural biotechnology* including their potential agricultural application in synthetic biology and for gene drive, taking into consideration the most recent developments in the agricultural sector. The explanations concerning questions 1 and 2 as specified below should be in scientific terms and should not examine legal issues.

Key characteristics of the various new techniques

SAM HLG is asked to provide an up-to-date overview on new techniques in agricultural biotechnology, whether ready to be used for commercial purposes or still at development stage, and on the key characteristics of each of these techniques (such as underlying molecular mechanism and products obtained). SAM HLG is also requested to describe potential agricultural applications of new techniques in the field of synthetic biology and gene drives.

Comparison with established techniques

SAM HLG is asked to explain the differences and similarities of each new technique as compared to 1) established techniques of genetic modification and 2) conventional breeding techniques. Where possible, differences and similarities should be identified in terms of safety for health and environment, possibilities for detection of the respective products, speed and cost to achieve the expected result and degree of maturity for field applications. Where published scientific evidence is insufficient, this should be explicitly stated.

In view of the timeframe in addressing questions 1 and 2 the explanatory note will be based on published literature reviews, scientific reports and existing published opinions which will be identified using a systematic and transparent procedure.

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7 SCENIHR, SCCS, SCHER (2014) Synthetic Biology I Definition, Opinion, September 2014. Available from: http://ec.europa.eu/health/scientific_committees/emerging/docs/scenihr_o_044.pdf
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SCENIHR, SCCS, SCHER (2015) Synthetic Biology III – Research priorities, Opinion, December 2015. Available from: http://ec.europa.eu/health/scientific_committees/emerging/docs/scenihr_o_050.pdf

In a second phase, SAM may subsequently be asked to supplement this work by describing expected trends in the next decade in agricultural biotechnology for plant and animal breeding, and for micro-organisms, and to anticipate forthcoming developments in the agricultural sector. This could include for example the anticipated development of further new techniques for synthetic biology and gene drive. The content and questions of the second phase will be specified based on the outcome of the first phase and in a second scoping paper.

In view of the likely public interest in the present topic, during the potential second phase, the Commission may involve other relevant expert groups to explore linked societal issues, which may include the aspects of public perception of agricultural biotechnology and of public engagement in the development of related scientific advice.

Further actors in support of the SAM HLG

EU academies and the wider scientific community may be consulted to collect scientific evidence and input.

Annex 2 - Methodology for the Evidence Review

This Annex sets out the systematic approach used for the collection, screening and analysis of evidence that has informed the development of the Explanatory Note (the Note) of the Scientific Advice Mechanism's High Level Group (SAM-HLG). The Note was produced by SAM-HLG in conjunction with academy fellows operating under the umbrella of the Coordination Support Action Science Advice for Policy by European Academies (SAPEA), funded under the current EU Framework Programme for Research and Innovation, Horizon 2020. Additional support was provided by the SAM Secretariat, staff members of SAPEA and the European Commission's Joint Research Centre (JRC).

Overview of the evidence base

Preliminary exploration of the topic confirmed the fast moving development of recent techniques used in agricultural biotechnology and their applications. This is matched by a large and rapidly expanding body of high quality evidence in the scientific literature (see Figure 10). These publications provided a sound basis for the production of an Explanatory Note setting out a description of, and comparison between, the available techniques. In order to complete the Note within the given time frame, as specified in the scoping paper for this Note (see Annex 1), mainly existing reviews, opinions and reports were used - which provided an efficient and rich source of evidence. Where very high volumes of literature were found, for example on the recent techniques of genome editing, particularly careful selection of the literature was required. The very fast developments in the area of genome editing, some of which are highly relevant for the subject of this Note, made it necessary to also consider some recent scientific journal articles on findings which were not yet covered by reviews, opinions or reports.

Approach

For the production of the Note, a project approach was taken with contributors being assigned clearly defined roles and responsibilities within

two groups, a review team and a steering group, operating under the leadership of the SAM-HLG.

The role of the Review Team (RT) was primarily to find and collate the information/evidence upon which the Note was produced. To do so the RT developed an evidence review 'protocol' that set out 'a priori' the method that was followed for the search and screening of the literature. Thereafter the Review Team performed the search and initial screening in accordance to the protocol. The RT provided additional assistance to the Steering Group with respect to the glossary and illustrations. The RT comprised Janusz Bujnicki and three invited staff members from SAPEA (Louise Edwards, Thomas Stehnken and Céline Tschirhart). Four members of the SAM Unit (Sigrid Weiland, Dulce Boavida, Stuart Kirk and Jeremy Bray) supported the activities of the RT. Additional, ad hoc support was provided by other members of the SAM-HLG and by technical experts from the JRC (Alexandre Angers, Mauro Petrillo and Amalia Muñoz Piñeiro).

The role of the Steering Group (SG) was to advise the RT, to contribute to the identification of evidence, make the final selection of source literature, and to conduct the bulk of the evidence synthesis to produce the draft and final Note. The SG, led by Janusz Bujnicki, comprised two other members of the HLG. Five academy fellows were invited to support the SAM-HLG as technical experts in the field (nominated by and supplied via the SAPEA consortium). An additional technical expert from JRC was also invited to support the SG. Throughout the production of the Note, four members of the SAM Unit supported the activities of the SG. A number of EC policy representatives were occasionally invited to attend parts of the SG meetings. The EC policy representatives provided background information on policy context and contributed to the clarification of some of the questions asked in the scoping paper.

The main sources of evidence used in the Note were as follows:

- A scientific literature search performed on four separate platforms/databases: Web of Science, SCOPUS, BIOSIS and Find-eR (EC's own database). The search strings were designed by topic experts and the

efficacy of the search strings was checked against their ability to find several known key references (previously identified by topic experts).

- Other sources of literature (references identified mostly by topic experts)

- Web using search engines (for illustrations and texts)

Largely due to time constraints, reviews, reports and existing scientific opinions were the main focus of the search and screening exercise. Only publicly accessible scientific evidence in English was used in the development of the Note.

All findings were combined in reference manager software and were screened and categorised for relevance. Algorithms were developed by JRC to assist with the screening and final selection of source literature, whereby key words were used to help identify the relevant publications to cover different aspects of the Note. In addition JRC provided analysis of the rates and volume of publications relating to the various techniques addressed by the Note (based upon a subset of findings) that usefully highlighted recent, significant developments associated with some of the NBT – an example of part of this analysis is shown in Figure 10.

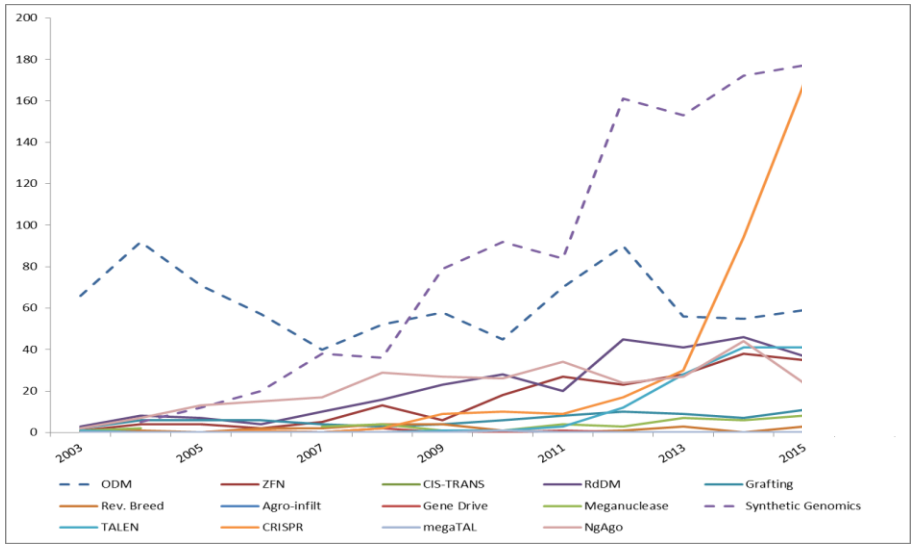

Figure 10 - Publications reviews over time by technique (Scopus) - Example output from analysis by JRC using TIM software

Source: Analysis courtesy of Mauro Petrillo and Alexandre Angers JRC (ISPRA) (http://www.timanalytics.eu/)

Results of the search and screening

In this way a large body of supporting evidence was retrieved and the most relevant information extracted to enable the development of the Note. The results of the search and screening process can be summarized in the form of a flow diagram (Figure 11).

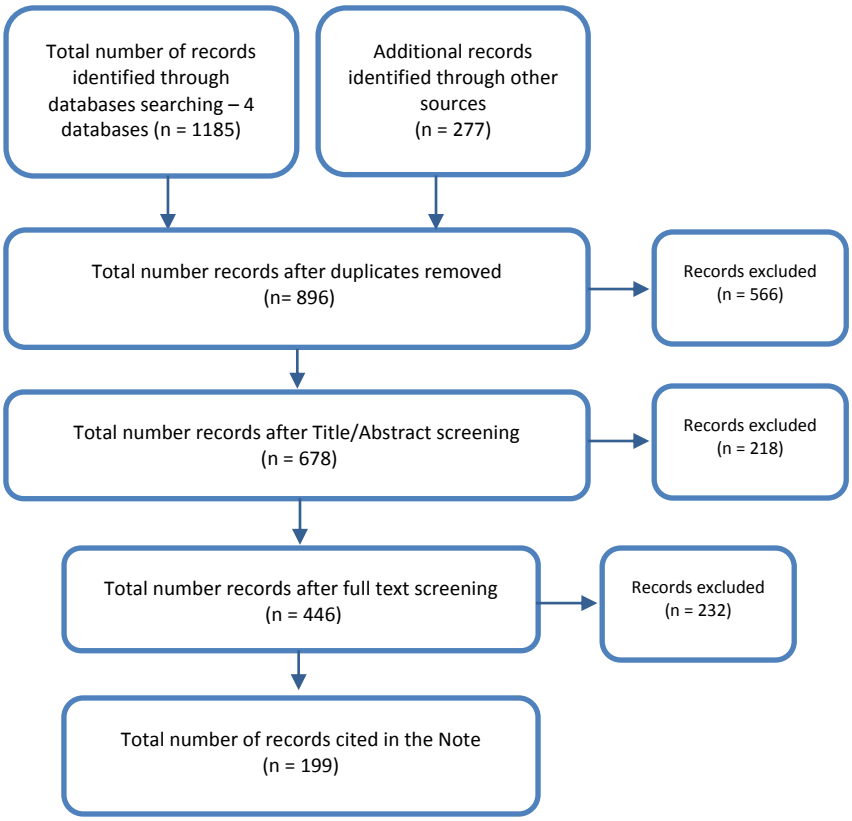

Figure 11 - Flow diagram of literature search results and screening

Source: SAM sec

Synthesis and completion

Upon conclusion of the evidence gathering process, the three High Level Group members tasked with leading the work, together with the experts nominated by SAPEA, fully developed the Note with the support of the SAM Secretariat. Initial findings were presented to the EU Commissioner for Health and Food Safety at a meeting of the SAM High Level Group on 24

March 2017. The Note was subsequently finalised, adopted by the SAM High Level Group and submitted to the European Commission on 28 April 2017.

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Annex 4 – Glossary

Glossary compiled from various sources, including consultations with experts.

Term	Explanation
Allele	Variant form of a given gene at the same genetic locus depending on the individual. Different alleles may correspond to different phenotypes.
Allogamy	Cross-fertilisation in plants
Autogamy	Self-fertilisation; pollination of the ovules of a flower by its own pollen
Amino acid	An organic compound. The genetic code involves 20 amino acids that are encoded by three nucleotide base pairs (codon) each. Chains of amino acids form proteins.
Annotation	A part of genome analysis carried out with the aid of computational tools that is typically performed before a genome sequence is deposited in databases and described in a scientific publication. A typical unit of annotation is the description of an individual DNA segment, usually a gene and its RNA and protein products, focused on the biological function (experimentally determined or predicted theoretically).
Abiotic	Devoid of life or living organisms.
Backcross	Crossing an individual with one of its parents or with the genetically equivalent organism. The offspring of such a cross are referred to as the backcross generation or backcross progeny.
Bacterium (pl.: bacteria)	Unicellular prokaryotic organisms, without a distinct nucleus. Major distinctive groups are defined by Gram staining. Also classified on the basis of oxygen requirement (aerobic vs anaerobic) and shape (spherical = coccus; rod-like = bacillus; spiral = spirillum; comma-shaped = vibrio; corkscrew-shaped = spirochaete; filamentous).
Base pair	A pair of purine and pyrimidine bases, in complementary strands of a double stranded nucleic acid. The bases are held together by specific hydrogen bonding. The base A pairs with T in DNA (with U in RNA); while G pairs with C in both DNA and RNA. The size of a double-stranded nucleic acid molecule is often given in terms of the number of base pairs it contains. See: nucleobase.
Biolistics bombardment (plasmid DNA)	A technique to generate transgenic cells, in which DNA-coated small metal particles (tungsten or gold) are propelled by various means fast enough to puncture target cells. Provided that the cell is not irretrievably damaged, the DNA is frequently taken up by the cell. The technique has been successfully used to transform animal, plant and fungal cells, and even mitochondria inside cells. *Synonym*: micro-projectile bombardment.
Biosecurity	A set of preventive measures designed to reduce the risk of transmission of infectious diseases in crops and livestock, as well as to avoid the spread of quarantined pests, invasive alien species, and living modified organisms.
Cell wall	An external tough structure which surrounds the membrane of some types of cells. It can be rigid or flexible. It is a characteristic feature of cells in plants, fungi, and most bacteria.
Centromere	The region of the chromosome to which the microtubules of spindle attach during cell division.

Chromosome	In eukaryotic cells, chromosomes are linear molecules of DNA, packaged by proteins (in particular histones) into a condensed structure called chromatin. They contain all or most of the genes, and are largely responsible for the differentiation and activity of the cell. Chromosomes are most easily studied in their contracted state, which occurs around the metaphase of mitosis or meiosis. Each eukaryotic species has a characteristic number of chromosomes. Prokaryotes (Bacteria and Archaea) typically contain only one circular chromosome, however also species with multiple and/or linear chromosomes are known. Viruses contain genetic material consisting of a single or double strand of DNA or RNA.
Cisgenesis	The introduction of a gene or genes into cells, as a result of artificial gene transfer between organisms that could otherwise be conventionally bred. Unlike in transgenesis, genes are only transferred between closely related organisms that belong to the same or sexually compatible species. The entire gene with its own regulatory elements is introduced. *See*: intragenesis, transgenesis.
Cloning, molecular cloning	1. The process of producing similar populations of genetically identical individuals. 2. The process of replicating a fragment of DNA.
Codon	A sequence of three nucleotides encoding an amino acid.
Colchicine	An alkaloid, obtained from the autumn crocus *Colchicum autumnale*, which blocks spindle formation (spindle is a protein structure that divides genetic material during cell division). When applied during mitosis, it prevents separation of chromosomes during anaphase. This property is used to achieve a doubling of the chromosome number. A further use is to halt mitosis at metaphase, the stage at which karyotypes are best viewed.
Copy number	The number of copies of a particular plasmid per microbial cell, or gene per genome.
Cotyledon	The primary leaf that constitutes a significant part of the embryo within the seed of a plant. The number of cotyledons present is a characteristic used by botanists to classify the flowering plants; species with one cotyledon are called monocotyledonous ("monocots") while species with two cotyledons are called dicotyledonous ("dicots").
CRISPR	*See:* SDN.
Cross-pollination	Application of pollen from one plant to another to effect the latter's fertilization.
Cultivar	A plant variety produced by selective breeding.
DNA	Abbreviation for deoxyribonucleic acid. DNA is a biological polymer that constitutes the genetic material of all known organisms, some organelles (including mitochondria and chloroplasts) and some viruses. In cells, DNA usually occurs in the form of a double helix formed by very long complementary strands arranged in an antiparallel way. *See*: base pair, genetic code, genome, nucleobase.
DNA inversions or translocations	Types of gross mutations in which a chromosome or large DNA fragment is inverted or in which a rearrangement occurs between different sites in the DNA molecule. Chromosomal translocations involve a transfer of a portion of one chromosome to a non-homologous location in another chromosome.
DNA Polymerase	An enzyme that synthesizes DNA molecules from deoxyribonucleotides. DNA polymerases are essential to DNA replication and to many processes that repair damage in DNA. During this process, DNA polymerase "reads" the existing DNA strand used as a template to create a new strand, which is complementary to the template (i.e. forms base pairs with the template). Thermostable DNA polymerases are used in the Polymerase Chain Reaction (PCR).

DNA sequencing	Procedures for determining the nucleotide sequence of a DNA fragment, i.e. the precise order of nucleotide residues (A, G, C, T) within a DNA molecule. Early DNA sequencing methods developed in 1970s were relatively slow, costly and laborious. Since the 1990s several new methods for DNA sequencing called "next-generation" or "second-generation" have been developed that allow for low-cost high-throughput sequencing of entire genomes. *See*: DNA, nucleobase, nucleotide.
Domain	The highest taxonomic rank. In one of the most commonly used taxonomic systems, living organisms are grouped into three domains: Archaea, Bacteria, and Eukaryota.
End Product	In the context of this Note: The final organism obtained by a breeding technique, such as a crop plant.
Endonuclease	An enzyme that cleaves a phosphodiester bond within a nucleic acid strand, generating a strand break. *See*: exonuclease, restriction endonuclease, side-directed nuclease.
Enzyme	A protein which, even in very low concentration, catalyses specific chemical reactions but is not used up in the reaction. Enzymes are classified into six major groups (1-6), according to the type of reaction they catalyse: 1. oxidoreductases; 2. transferases; 3. hydrolases; 4. lyases; 5. isomerases; 6. ligases. Generally enzymes are named by the addition of the suffix -ase to the name of their substrate, and are classified by a standard numerical system: the Enzyme Commission (EC) number.
Epigenesis	Describes the developmental process whereby each successive stage of normal development is built up on the foundations created by the preceding stages of development; an embryo is built up from a zygote, a seedling from an embryo, and so on.
Epigenetics	Describes information encoded in chromosomes, but not directly in the DNA sequence, which contributes to the determination of stable, heritable phenotype, along with the genotype and environmental factors. Epigenetics often refers to changes in a chromosome such as chemical modification of DNA or histone molecules that affect gene activity and expression. These changes are often triggered by environmental factors. The term "epigenome", in analogy to the term genome, refers to the overall epigenetic state of a cell, and epigenomics refers to global analyses of epigenetic changes across the entire genome or in the whole cell.
Eukaryote	One of the major evolutionary clades (domains), characterized by having the nucleus enclosed by a membrane, and possessing chromosomes that undergo mitosis and meiosis. Eukaryotic organisms include animals, plants, fungi, algae, and various microorganisms. *See*: prokaryote.
Exogenous	Produced outside of; originating from, or due to, external causes. *Opposite*: endogenous.
Exogenous DNA	DNA originating outside the organism of concern or under investigation which can be introduced naturally or by technological intervention.
Exonuclease	An enzyme that cleaves nucleotides one at a time from the end (exo) of a nucleotide chain. *See*: endonuclease.
Filler DNA	Portions of DNA that can accidentally serve as templates during repair of double stranded breaks (DSBs) by the homology-directed repair (HDR) mechanism.
Flow cytometry	Automated measurement on large numbers of individual cells or other small biological materials, made as the cells flow one by one in a fluid stream past optical and/or electronic sensors. A sensor detecting the scattered or emitted light measures the size and molecular characteristics of individual cells.

Founder animal	An organism that carries a transgene in its germ line and can be used in mating to establish a pure-breeding transgenic line, or one that acts as a breeding stock for transgenic animals.
Gamete	A haploid (see polyploidy) cell that can fuse with another complementary gamete in sexual reproduction, to produce a zygote.
Gene expression	The process by which a gene produces RNA, which (in the case of protein-coding RNAs) is subsequently translated into protein, and thereby exerts its effect on the phenotype of an organism. See: RNA.
Gene pool	The sum of all genetic information in a breeding population at a given time.
Genetic diversity	Total number of genetic characteristics in the genetic makeup of a species; genetic variability: tendency of genetic characteristics to vary; with more variation, individuals in a population possess a larger number of gene variants (alleles). See: allele.
Genetic linkage	The tendency of genetic elements which are located closely together on a chromosome to be inherited together during the meiosis phase of sexual reproduction.
Genome	1. The entire complement of genetic material (including coding and non-coding sequences) present in a cell of an organism, a virus, or an organelle. 2. The complete set of chromosomes (hence of genes) inherited as a unit from one parent.
Genotype	The genotype corresponds to the DNA sequence of a cell, and therefore of an organism or individual, which determines, together with epigenetic and environmental factors, stable and heritable characteristics (phenotype) specific for that cell/organism/individual.
Germplasm	1. Living genetic resources (an individual organism, group of individuals, seeds, tissues etc.) that represent a genotype, variety, species or culture, which are maintained as a collection for the purpose of animal and plant breeding, preservation, and research uses. 2. Original meaning, now no longer in use: the genetic material that forms the physical basis of inheritance and which is transmitted from one generation to the next by means of the germ cells.
Heterozygote	An individual with non-identical alleles for a particular gene or genes. The condition is termed "heterozygous". *See*: homozygote.
Hexaploid	An organism containing six sets of chromosomes (see also polyploidy)
Histone	A highly alkaline protein found in eukaryotic cell nuclei. Histones are the main protein components of chromatin, they package and order the DNA into structural units called nucleosomes. They play a major role in regulating gene expression.
Homology-directed repair	Abbreviated as HDR. A cellular process, where a DNA double-strand break (DSB) is repaired. The most common form of HDR is homologous recombination. HDR can only be used when there is a similar or identical DNA fragment present that can be used as a repair template. Alternatively, another process called non-homologous end joining (NHEJ) can take place instead without the use of homologous DNA piece.
Homozygote	An individual that has the same allele for a given gene on its two homologous chromosomes. The condition is termed "homozygous". *See*: heterozygote.
Inbreeding	Is a technique used in selective breeding in plants or animals to substantially reduce heterogeneity. It involves production of offspring from the mating or crossing of organisms that are closely related genetically. In plant breeding, inbred lines are used as stocks for the creation of hybrid lines to make use of the effects of heterosis (the phenotypic superiority of a cross over its parents). Inbreeding in plants also occurs naturally in the form of self-pollination.
Intermediate Product	In the context of this Note: A modified organism produced as an intermediate step in the production of an end product

Intragenesis	The introduction of a gene or genes into cells, as a result of artificial gene transfer between organisms that could otherwise be conventionally bred. Unlike in transgenesis, genes are only transferred between closely related organisms that belong to the same or sexually compatible species. The inserted DNA can be a new combination of regulatory or coding DNA fragments from the same or sexually compatible species. *See*: cisgenesis, transgenesis.
In vitro, in vivo	Terms used to describe processes outside or inside of living organisms. Usually with reference to experiments conducted in test tubes or culture dishes on parts of living or dead organisms (in vitro) or on, or in whole living organisms (in vivo).
Marker assisted selection	The use of DNA markers to improve selection in a population. The markers will be closely linked to one or more target loci, which may often be quantitative trait loci.
Metabolite	A low-molecular-weight biological compound that is usually synthesized enzymatically.
Microbiome	An ecological community of microorganisms (including bacteria, archaea, protists, fungi and viruses) found in and on a multicellular eukaryotic organism.
Mitosis	A phase of the cell cycle which involves the splitting of replicated chromosomes, and the division of the cytoplasm to produce two genetically identical daughter cells. On the basis of the appearance of the chromosomes, it is separated into five stages: interphase, prophase, metaphase, anaphase and telophase.
Monocotyledon	Abbreviated as Monocot. A flowering plant whose embryo has one cotyledon. Examples are cereals (corn, wheat, rice etc.), banana, and lily.
Mosaic	An organism or part of an organism that is composed of cells with different origin.
Mutation	A permanent change to the nucleotide sequence of the genome of an organism, a virus, or another genetic element.
Non-homologous end joining	Abbreviated as NHEJ. A cellular process, where a DNA double-strand break (DSB) is repaired by direct ligation of the break ends. As opposed to homology-directed repair, it can be used in the absence of a homologous repair template. NHEJ can repair the break accurately, but imprecise repair leading to loss of nucleotides can also occur. When multiple breaks occur and multiple ends coincide, NHEJ can join 'wrong' ends, i.e. base pairs which were not previously joined in the wild-type with each other.
Nucleic acid	A macromolecule consisting of polymerized nucleotides. In living organisms two types are commonly found, DNA and RNA. Nucleic acids may be linear or circularized, and single- or double-stranded.
Nucleobase	Often called a base. A heterocyclic aromatic organic chemical compound, which serves as a building block of nucleotides that form nucleic acids (DNA or RNA). The chemical structure of cytosine (C), uracil (U), and thymine (T) bases is derived of pyrimidine and contains a single aromatic ring. The chemical structure of adenine (A) and guanine (G) bases is derived of purine. See: base pair, nucleoside, nucleotide, DNA, RNA.
Nucleoside	A compound formed by a chemical bonding of a nucleobase with a sugar molecule: ribose (ribonucleoside) or deoxyribose (deoxyribonucleoside). Nucleosides containing the bases adenine, guanine, cytosine, thymine, and uracil, are called adenosine, guanosine, cytosine, thymidine, and uridine, respectively. *See*: nucleobase, nucleotide.

Nucleotide	A basic building block of nucleic acids, formed by the bonding of a nucleoside with one or more phosphate groups.. When the sugar is ribose, the nucleotide is a ribonucleotide; when it is 2-deoxyribose, the nucleotide is a deoxyribonucleotide. RNA and DNA are polymers of, respectively, ribonucleoside 5'-monophosphates and deoxyribonucleoside 5'-monophosphates. Nucleotides containing the bases adenine, guanine and cytosine (A, G, C) occur in both DNA and RNA; thymine (T) occurs only in DNA, and uracil (U) only in RNA. Ribonucleoside mono-, di-, and triphosphates for which a specific base is not assigned are abbreviated NMP, NDP, and NTP, while deoxyribonucleoside mono-, di-, and tri-phosphates are abbreviated dNMP, dNDP, and dNTP. Otherwise, the "N" is replaced by the base letter abbreviation. *See*: base pair, nucleobase, nucleoside.
Off-target mutation	Any change in the genome with respect to a defined wild type, made to a genetic sequence in another location than the desired target. Off-target mutations can occur in sequences identical or similar to the target. These mutations can be silent (i.e. cannot be associated with any change in phenotype), either because the DNA sequence affected is in the non-coding part of the genome, or because the specific change does not alter the function of a coding sequence.
Oligonucleotide	A nucleotide oligomer. Often synthesized chemically for use in mutagenesis or as primers for *in vitro* DNA synthesis. *See*: polymerase chain reaction.
Phenotype	The visible appearance of an organism (with respect to one or more traits) which reflects the interaction of a given genotype with a given environment. See: genotype.
Pleiotropic effects	Effects resulting from changes to a gene which is related to two or more seemingly unrelated phenotypic traits.
Pluripotency (see also totipotency)	The ability of a stem cell to give rise to several different cell types.
Polymerase Chain Reaction	Abbreviated as PCR. A widespread molecular biology procedure that allows the production of multiple copies (amplification) of a specific DNA sequence, provided that the base pair sequence of each end of the target is known. It involves multiple cycles of DNA denaturation, primer annealing, and strand extension, and requires a thermostable enzyme DNA polymerase, deoxyribonucleotides, and specific oligonucleotides (primers).
Polyploidy	Polyploid organisms contain more than two homologous sets of chromosomes. Note also haploid (containing a single set of unpaired chromosomes) and diploid (containing two complete sets of chromosomes, one from each parent).
Population genetics	The branch of genetics that deals with frequencies of alleles and genotypes in breeding populations.
Primordial germ cell	Abbreviated as PGC. The primary undifferentiated sex cell type that will differentiate towards gametes: spermatozoa or oocytes. With the development of stem cell biology and differentiation protocols, PGC can be obtained from pluripotent stem cells.
Prokaryote	A unicellular organism that lacks a membrane-bound nucleus, or any other membrane-bound organelles. Prokaryotes comprise two evolutionary clades (domains), Archaea and Bacteria. In contrast, species with membrane-bound nuclei and organelles are placed in the domain Eukaryota.
Protein	A large biological molecule. Proteins consist of one or more long chains of amino acids, the sequence of which is dictated by the nucleotide sequence of the genes, by which they are encoded. The sequence of amino acids specifies the spatial folding of the protein chain and determines its activity. Proteins perform various functions within organisms, such as enzymatic catalysis of chemical reactions (including synthesis of DNA and RNA), response to stimuli, and transport of molecules.

Protoplast	A bacterial or plant cell for which the cell wall has been removed either chemically or enzymatically, leaving its cytoplasm enveloped by a plasma membrane. Protoplasts are spherical and smaller than the elongate, angular shaped and often vacuolated cells from which they have been released.
Recombinant	A term used in both classical and molecular genetics. Typically used as an adjective, *e.g.* recombinant DNA. 1. In classical genetics: An organism or cell that is the result of genetic recombination. 2. In molecular genetics: A hybrid molecule made up of DNA obtained from different organisms.
Restriction enzyme	An enzyme (endonuclease) that cuts DNA at or near specific nucleotide sequences known as restriction sites. Such enzymes are found in nature in most prokaryotic species and can provide defence against exogenous DNA *e.g.* from invading viruses. They are routinely used for DNA manipulation in biotechnological applications.
Retrovirus	An RNA-containing virus which uses a host cell's machinery to transcribe its RNA into DNA, which is then incorporated into the host genome and transcribed, resulting in the production of more viruses.
Ribonucleoprotein	A molecular complex that consists of ribonucleic acid and protein molecules.
RNA	Abbreviation for ribonucleic acid. RNA is an essential biological polymer involved in various biological roles in coding, decoding, regulation, and expression of genetic information. In cells, RNA like DNA is assembled as a chain of nucleotides, but unlike a typically double-stranded DNA, RNA usually comprises a single-strand. Many viruses encode their genetic information using an RNA genome (which can be single- or double-stranded). There are many different types of functional RNA molecules. Messenger RNAs (mRNAs) encode proteins. RNAs that do not encode proteins are called "non-coding" and they play various roles within cells such as catalyzing biological reactions, regulating gene expression, or sensing and communicating responses to cellular signals. For example ribosomal RNAs (rRNAs) and transfer RNAs (tRNAs) govern the process of protein synthesis on the mRNA template. Guide RNA molecules are used by site-directed nucleases from the CRISPR-Cas system to target these nucleases for DNA cleavage. *See*: DNA, genome, nucleobase.
Sequence	The linear order of nucleotides along a DNA or RNA molecule. *See:* DNA sequencing, genome.
Silencing	Loss of gene expression either through an epigenetic alteration in the DNA sequence of a structural gene, or its regulatory region; or because of interactions between its transcript and other RNAs present in the cell.
Site-directed nuclease	Abbreviated as SDN. An enzyme (endonuclease) that creates site-specific double-strand breaks (DSBs) at defined sequences. SDN typically recognises a specific DNA sequence and "cleaves" DNA within such a sequence or nearby. The recognition of the DNA target can be achieved on a molecular level by the protein molecule (in protein-directed SDNs) or by a guide RNA molecule (in RNA-directed SDNs). Examples of protein-directed SDNs found in nature and used in various biotechnological applications include restriction enzymes and meganucleases. Various artificial SDNs were developed by attaching a nuclease domain for DNA cleavage, with a DNA binding domain for recognition of a specific DNA sequence, including zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs). The challenge in application of protein-directed SDNs is that for each DNA target sequence a different protein must be developed, which is typically a lengthy process that is not always successful. RNA-directed SDNs found in nature are exemplified by the CRISPR-Cas system. They consist of a protein module (including a nuclease) which is bound to a guide RNA, the sequence of which targets the nuclease to the complementary DNA sequence in the genome. The development of an RNA-directed SDN

	for a particular DNA target sequence requires only a specific guide RNA to be developed, which is much easier and more efficient than in the case of protein-directed SDNs known to date. See: nuclease
Somatic	Referring to cell types, structures and processes other than those associated with the germ line.
Somatic cell	Cells not involved in sexual reproduction, i.e. not germ cells.
Somatic hybridisation	Naturally occurring or induced fusion of somatic protoplasts or cells of two genetically different parents. The difference may be as wide as interspecific. Wide synthetic hybrids formed in this way (i.e. not via gametic fusion) are known as cybrids. Not all cybrids contain the full genetic information (nuclear and non-nuclear) of both parents.
Southern hybridization	A procedure, also called Southern blotting, in which an isolated, labelled segment of DNA is hybridized to DNA restriction fragments separated by electrophoresis. It is used for detection of a specific DNA sequence in DNA samples.
Stable transformation	A process by which the genetic material carried by an individual cell is stably altered by the incorporation of exogenous DNA.
Substrate	1. A compound that is altered by an enzyme. 2. Food source for growing cells or micro-organisms. 3. Material on which a sedentary organism lives and grows.
T-DNA	Transfer DNA of the tumour-inducing (Ti) plasmid of some species of *Agrobacterium* bacteria. The T-DNA can be transferred from the bacterium into the host plant's nuclear DNA genome. *Agrobacterium*-mediated transfer of engineered T-DNA can be used as a tool in biotechnology to generate transgenic plants carrying a foreign gene.
TECCDNA or TECCRNA	Transiently expressed CRISPR/Cas9 DNA or transiently expressed in vitro transcripts of Cas9-coding sequence and guide RNA.
Tissue culture	The *in vitro* culture of cells, tissues or organs in a nutrient medium under sterile conditions.
Totipotency (see also pluripotency)	The ability of a cell or tissue to be induced to regenerate into a complete organism.
Trait (phenotypic)	A distinct variant of a phenotypic characteristic of an organism or a cell. It may be inherited, and modified environmentally. For example, a colour is a character of a flower petals, while white or red are traits.
Transduction	The process of DNA transfer into cells with the use of a virus or a viral vector (the transferred DNA is exogenous with respect to the virus).
Transfection	The process of introducing isolated nucleic acid (DNA or RNA) into eukaryotic cells, in which the introduced nucleic acid is typically intended to change the phenotype of the recipient organism in a predictable manner.
Transformation	1. The process of the direct uptake of nucleic acid in bacteria and non-animal eukaryotic cells from their surroundings. Transformation occurs naturally in some species of bacteria, and can also be done artificially. Bacteria that are capable of being transformed, whether naturally or artificially, are called competent. In the process of artificial transformation, the introduced nucleic acid is typically intended to change the phenotype of the recipient organism in a predictable manner. In relation to animal cells, transformation has a special different meaning (see 2. below), and in animal cells a process analogous to transformation is usually called transfection. 2. The conversion, by various means, of cultured animal cells from controlled to uncontrolled cell growth, typically through infection with a tumour virus or transfection with an oncogene; also in general progression of animal cells to a cancerous state.
Transgene	An exogenous gene used to transform an organism
Transgenesis	The introduction of an exogenous gene or genes into cells, which leads to the transmission of the input gene (transgene) to successive generations.

Trophic	Relating to nutrition.
Taxonomy	A branch of science that encompasses the description, identification, nomenclature, and classification of groups of biological organisms on the basis of shared characteristics, and giving names to those groups.
Virus	An infectious agent composed of a protein capsule and a nucleic acid core (DNA or RNA), which is dependent on a host organism for replication.
Wild relative or wild type	The most frequent allele or genotype found in nature, or a specified organism against which mutants are defined.

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This Explanatory Note on New Techniques in Agricultural Biotechnology (new breeding techniques) responds to a request made to the High Level Group of Scientific Advisors by Vytenis Andriukaitis, European Commissioner for Health and Food Safety.

The Note provides a scientific and technical description of a wide range of breeding techniques used in agriculture in plants, animals and microorganisms, which are grouped under umbrella terms that reflect both historic and recent developments in breeding techniques, namely: conventional breeding techniques, established techniques of genetic modification, and new breeding techniques.

The Note compares the various techniques according to a variety of criteria including: nature and frequency of unintended effects, the maturity of the technique, the speed and cost with which the desired outcome can be achieved, and the ability to detect and identify changes in end products resulting from the employment of these techniques. Aspects related to safety are also briefly considered.

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