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Supporting document 1

Safety assessment: full technical report

P1055 – Definitions for gene technology and new breeding techniques

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Executive summary

The Australia New Zealand Food Standards Code (the Code) contains definitions that determine what foods are genetically modified (GM) food and therefore require pre-market safety assessment and approval. These definitions were based on established GM techniques in use when the GM food standard was adopted in 1998. These techniques typically result in the transfer of foreign DNA.

Over the last decade, a variety of new breeding techniques (NBTs) have increasingly been applied to the production of food. The emergence of these techniques has generated uncertainty about the regulatory status of food derived using NBTs (NBT food), because these techniques often result in genetic changes that are more similar to those from conventional breeding rather than from established GM techniques. Unlike GM food, conventional food does not require pre-market safety assessment and approval before it can be sold.

To address the uncertainty, FSANZ began a proposal to revise and update the GM food definitions in the Code to:

- make them clearer and better able to accommodate food produced by existing, emerging and future genetic technologies, and
- ensure NBT foods are regulated in a manner commensurate with the risk they may pose.

Before the GM food definitions can be revised, it must first be determined whether justification exists for subjecting each new NBT food to pre-market safety assessment, similar to GM food.

To help inform a decision about how NBT food should be regulated, FSANZ applied an assessment approach that is typically used in GM food safety assessment. That approach relies on comparisons to conventional food, which serves as the benchmark for safety.

In applying this comparative approach, FSANZ considered whether:

- conventional food is a suitable benchmark against which to compare NBT food, and
- similarity in product characteristics between a NBT food and a conventional food indicate they are also equivalent in terms of risk.

To address these questions, the safety assessment focussed on two key aspects:

- The history and origins of conventional food, including the types of genetic changes that have occurred through both natural means as well as through conventional breeding. The assessment also examined how specific standard breeding practices, such as backcrossing, screening and selection, may contribute to the production of safe food.
- Possible genetic changes that may arise from NBTs compared to conventional breeding methods. The assessment also considered whether there were unintended changes specific to NBTs that would invalidate the comparison to conventional food.

In response to these investigations, the assessment found that substantial genetic changes exist in all organisms used for food. Breeders rely on this genetic variation to produce food organisms or food with improved characteristics, for example higher yield or better flavour. The standard practice of screening and selecting for improved characteristics also serves to identify organisms with undesirable characteristics, some of which may impact food safety. Organisms with undesirable characteristics will be removed from the breeding program and will not enter the food supply. While both beneficial and harmful changes can and do occur, most genetic changes are neutral and do not change the organism or the food in any meaningful way, or significantly impact food safety. Conventional food thus has a long history of safe use. The assessment concluded that conventional food is a suitable benchmark against which to compare NBT food.

The assessment also found that a large variety of genetic changes can be generated using NBTs. In some cases these changes will be identical to those introduced using conventional breeding, while in other cases they may resemble changes introduced using established GM techniques. In terms of unintended changes, these are an expected outcome of all methods for modifying genomes, and those that arise from NBTs are no different to those from conventional breeding and GM techniques. As a result, some NBT food will be similar in characteristics, and in many cases identical, to conventional food.

When NBT food is equivalent in product characteristics to conventional food with a history of safe use, the NBT food can also be considered equivalent in risk. The same is true for certain refined ingredients derived from GM food, i.e. those products without novel DNA and novel protein in the food for sale and with characteristics identical to conventional products.

This conclusion supports the exclusion of NBT foods and refined ingredients considered equivalent in risk to conventional food, from the requirement for pre-market safety assessment as a GM food.

Table of contents

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Glossary

Term	Description
Backcrossing	The mating of an organism to a parent or an individual genetically similar to the parent.
Cell and tissue culture	The practice of growing plant, animal or microbial cells, or plant or animal tissues in the laboratory.
Cisgenesis	DNA from the same or a cross-compatible species is inserted into the genome of an organism without altering the inserted DNA sequence or configuration.
Conventional breeding	Use of traditional methods for developing new traits in an organism, without involving gene technology.
Cross breeding	The mating of different species, breeds or varieties.
DNA	**D**eoxyribo**n**ucleic **a**cid is the hereditary material for most living organisms. DNA is present in cells as two strands (double stranded) composed of a series of nucleotides.
DNA polymerases	A group of enzymes that copy DNA.
Double-stranded DNA break	When both strands of the double-stranded DNA molecule are cut and can be separated.
Foreign DNA	DNA obtained from a different species.
Gene technology	Recombinant DNA techniques that alter the heritable genetic material of living cells or organisms (specified in Standard 1.5.2). May also be called GM techniques.
Genetic modification	The process of altering the DNA of an organism.
Genetically modified organism (GMO)	An organism whose genome has been modified using gene technology.
Genome	The complete set of genetic material in a living cell or organism.
Genome editing	A group of techniques that make precise changes at targeted locations in the genome of an organism.
GM food	Food derived from organisms that have been modified using gene technology.
Helicases	Enzymes that unwind and separate the two strands of DNA. Helicases are required for copying DNA and for transcribing DNA into RNA.
Indel	Small nucleotide **in**sertion and/or **del**etion.

Intragenesis	DNA from the same or cross-compatible species is rearranged before being inserted into the genome of an organism.
Inversion	The reversal of a section of DNA within the genome.
Ligases	A group of enzymes that join (ligate) two strands of DNA together.
Mutagenesis	The act of introducing a mutation into the genome.
NBT food	Food from an organism modified using a new breeding technique.
New breeding techniques (NBTs)	A wide range of new techniques used to modify the genomes of plants, animals and microorganisms.
Nucleases	A group of enzymes, found in all cells, that cut DNA.
Nucleotide	The basic structural unit of DNA. For all living organisms, there are four types of nucleotides: adenine (A); guanine (G); cytosine (C) and thymine (T).
Null segregant	Progeny that have not inherited a genetic modification.
Point mutation	A change to a single nucleotide in DNA.
Recombinant DNA techniques	*In vitro* laboratory techniques that are used to recombine or join DNA from two or more sources.
GM rootstock grafting	Joining the vegetative (upper) part of a compatible plant variety to the rootstock of a GM plant.
Scion	The vegetative upper part of a plant that is joined to a rootstock.
Trait	A distinguishable characteristic belonging to an organism, such as eye colour.
Transgenesis	Transfer of DNA between two different species, unable to normally breed or exchange DNA.
Translocation	Occurs when part of a chromosome breaks off and reattaches to another part of the same or a different chromosome.

1. Introduction

Over the last decade, a variety of new breeding techniques (NBTs) have increasingly been used to modify the genomes of organisms used for food. These techniques are different from more established methods used to produce GM food because they often do not result in foreign DNA remaining in the final organism used for food. Instead, the genetic changes that typically result from the application of NBTs more closely resemble those introduced using conventional approaches for modifying genomes or that occur naturally.

The different outcomes that may arise from NBTs compared to established GM techniques has resulted in a regulatory problem. The current definitions in the Australia New Zealand Food Standards Code (the Code)[1], which determine what is a GM food and therefore requires pre-market safety assessment and approval, were based on the established techniques that existed when the GM food standard was introduced[2]. These techniques typically result in the transfer of foreign DNA between organisms. The applicability of these definitions to the techniques that have emerged since that time is unclear.

The other aspect to this problem relates to the similarity in outcomes between NBTs and conventional approaches for modifying genomes. In Australia and New Zealand, and most other countries around the world, food derived using conventional approaches (conventional food) is not subject to pre-market safety assessment and approval because it is well accepted such food is safe.

This has raised a question about whether it is appropriate and scientifically justified to subject food derived using NBTs (NBT food) to pre-market safety assessment and approval if the food is no different to conventional food. On the one hand, the techniques are relatively new and there is less familiarity with them from both a scientific and a regulatory perspective. This has led to a diversity of views in the community about the acceptability and safety of NBT food, and how it should be regulated. On the other hand, NBT food is said to closely resemble, or in some cased be indistinguishable from, conventional food which is well accepted as safe.

FSANZ considered these issues in the *Review of Food derived using New Breeding Techniques*, the final report of which was released in December 2019[3]. That review found there may be a case, based on risk, for some NBT foods to be excluded from pre-market safety assessment. In making this finding, it was noted the similarity of NBT food to conventional food would be a relevant consideration in deciding whether a pre-market safety assessment of a NBT food was warranted. FSANZ undertook to further examine this issue before reaching any final conclusions.

Making comparisons to conventional food as a basis for establishing safety is a concept that is routinely applied to GM food. In this case, the conventional (non-GM) counterpart food serves as the benchmark for what is considered safe (Codex 2009; FSANZ 2019). In the case of GM food, foreign DNA has usually been introduced and this foreign DNA typically results in the expression of a new protein or other substance which confers a specific new trait on the organism. The purpose of the comparison is to: identify any differences; further examine those differences to see if they raise any safety issues; and finally to establish whether the GM food is equivalent to its conventional counterpart in terms of safety, recognising it may not be fully equivalent in terms of the final food characteristics because of the new trait introduced.

[1] '*food produced using gene technology*' and '*gene technology*'
[2] A dedicated GM food standard was adopted in 1998.
[3] www.foodstandards.gov.au/consumer/gmfood/Documents/NBT%20Final%20report.pdf

The question for FSANZ to consider through this assessment is whether a comparative approach could be used as a basis for deciding whether certain NBT foods could be excluded from pre-market safety assessment. In other words, whether sufficient evidence exists to support certain NBT foods being considered the same as conventional food for regulatory purposes.

To aid these considerations, the assessment has focussed on cataloguing the extensive genetic differences that exists in organisms which are used for the conventional food supply and comparing these to the genetic changes that are possible using NBTs. This analysis has taken into account spontaneous/naturally occurring genetic changes, intentional genetic changes as well as unintended genetic changes. This information has been used to consider two key questions:

1. Is conventional food a suitable benchmark against which to compare NBT food?

2. Does similarity in product characteristics between a NBT food and a conventional food indicate they are also equivalent in terms of risk?

The outcome of this assessment will be used to inform the proposed approach for amending the definitions for 'gene technology' and 'food produced using gene technology', and consider whether all NBT food should be subjected to pre-market safety assessment, similar to GM food.

2. Conventional food

Virtually all human food is derived from domesticated species. Domestication occurs when wild varieties are exposed to new selective environments associated with human propagation and use (reviewed in Purugganan and Fuller 2009). The domestication of wild plants and animals, and adapting them for human food use by selection, commenced about 12,000 years ago (reviewed in Purugganan 2019). Fruit and cereals were among the first plants to be domesticated, while the domestication of goats, pigs, cattle and sheep began about 10,000 years ago (reviewed in MacHugh et al. 2017). Microbial domestication occurred slightly later (ca. 6,000 years ago) when humans first started consuming fermented foods.

Since that time, a variety of different methods and approaches have been used to continuously adapt and improve the characteristics of organisms for human food use. In this context, improved characteristics include those that enable increased or more efficient food production, such as better abiotic or biotic[4] stress tolerance, higher fertility in sexually reproducing organisms, greater uniformity, faster growth and maturation. Other enhanced characteristics include those that change the food itself, such as improved nutrient content, reduced toxin content and bitterness, delayed ripening or longer shelf life.

Such improvements are possible because of the inherent genetic variation that exists in all organisms.

2.1 Natural genetic variation

Genomes of organisms are dynamic and evolve over time, as they are subject to selection. The main contributors to genetic variation include errors in DNA integrity checking/repair or replication; changes to ploidy[5]; mobile genetic elements; and sexual reproduction/meiotic

[4] Abiotic stress is stress imposed on an organism by physical or chemical factors in the environment, e.g. salinity, sunlight, drought. Biotic stress is stress imposed on an organism by biological factors such as an insect pest, a parasite or a virus.
[5] The number of complete sets of chromosomes in a cell.

recombination. A summary of these are provided below with further details and examples in Appendix 1.

Mutations that change DNA sequences are common and contribute to genetic variation in organisms, which in turn drives evolution. Mutations are caused by a variety of factors including biological, chemical and physical agents. It has been estimated that approximately 10-50 double-stranded DNA breaks occur within each mammalian cell per day (reviewed in Cannan and Pederson 2016). Such events can lead to a variety of genetic alterations including small deletions or larger DNA rearrangements. While all organisms have natural cellular mechanisms to repair DNA, this process is not perfect and can lead to heritable mutations. Accumulation of these 'spontaneous' or 'natural' mutations over time has led to significant genetic variation in populations.

Over the course of evolution, whole genome duplication (resulting in polyploidy) has occurred and persisted in most eukaryotic lineages, including animals, plants and fungi, although it most commonly occurs in plants (reviewed in Albertin and Marullo 2012; Leitch and Leitch 2008). Such genome duplication events can cause a large variety of genetic change including gene loss, gene silencing, redirection of gene function, chromosomal rearrangements, epigenetic changes[6] and changes in the activity of mobile genetic elements (reviewed in Soltis and Soltis 2021). Increases in ploidy widen the genetic base for the evolution of organisms.

Genetic variation and breeding

Genetic variation is fundamental to the evolution and survival of plants, animals and microbes.

Breeders rely on genetic variation in populations to generate new traits. Populations are screened to identify individuals with beneficial traits which are then selected and used in further breeding.

If the genetic change leading to a beneficial trait is known, NBTs can be used to directly introduce the change into other individuals. This can significantly shorten the breeding process.

Mobile genetic elements are a source of genetic variation in organisms. They are pieces of DNA that are able to move within a genome or between genomes using a variety of mechanisms. Transposons are one example of mobile genetic elements and their insertion in different parts of the genome can cause insertional effects. This includes changes to genome architecture and size; chromosomal rearrangements; alteration of gene expression and mobilisation of endogenous sequences (reviewed in Fambrini et al. 2020). Insertional effects such as these are natural and common in a wide range of food organisms.

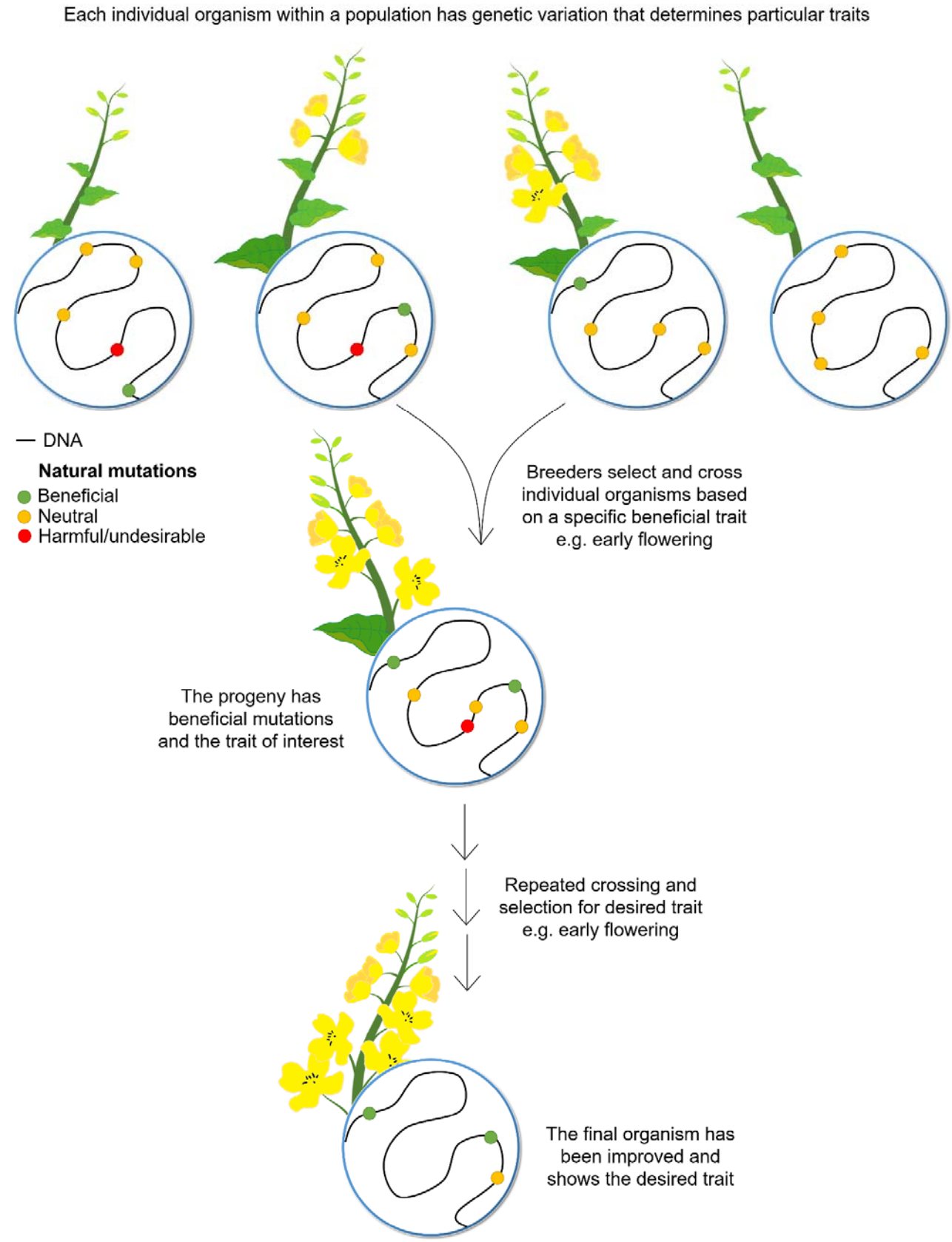
Another source of genetic variation is sexual reproduction. This process leads to genetic variation in the offspring and hence in populations overall. Genetic variation stems from independent assortment and crossing over of chromosomes during the process of sexual reproduction. This involves double-stranded DNA breaks and repair, as well as the transfer of large numbers of genes between chromosomes. The process of cross-breeding animals or plants exploits this process to introduce new genetic variation.

2.2 Conventional approaches for genetic improvement

A high level of genetic variation is fundamental to food improvement, and for centuries, conventional breeding has harnessed the inherent genetic variation of organisms to select for particular characteristics (Figure 1). More recently developed conventional breeding approaches have been used to artificially increase the genetic variation of organisms (see

[6] Epigenetic changes are modifications that are associated with DNA but without altering the DNA sequence, e.g. DNA methylation.

Figure 1. *Selection of beneficial and removal of harmful genetic variation in a population*



Appendix 2 for more information). Conventional breeding methods include: selection and propagation, cross-breeding, mutagenesis; and various *in vitro* techniques. These approaches can drastically change the genomes and characteristics of animals, plants and microorganisms for food use.

Early domestication saw wild plants and animals being selected for beneficial characteristics. For example, a single domestication event involving the wild grass-like plant teosinte ultimately gave rise to the corn varieties that exist today (Figure 2). The hybridisation of partially domesticated species with wild relatives also dramatically accelerated the diversification of food organisms, the evolution of genes and the emergence of new combinations of characteristics (reviewed in Gregory 2009; MacHugh et al. 2017; Meyer and Purugganan 2013; Zhang et al. 2019). Cross-breeding, involving the deliberate crossing of sexually compatible plants or animals to combine superior characteristics, came later.

Figure 2. *Domestication of corn leading to a marked increase in ear and seed size[7]. Ears from i. teosinte; ii. hybrid between teosinte and maize; and iii. Maize*

In cross-breeding, genetic variation is increased primarily through chromosome assortment and crossovers, but also through point mutations, chromosomal rearrangements (e.g. inversions, translocations or deletions), whole genome duplication and transposon activation (reviewed in Gregory 2009). The practice of wide crossing – the hybridisation of distantly related varieties that do not normally sexually reproduce with each other – results in new hybrid varieties. For example, triticale (a cereal grain used primarily as livestock feed) is the result of a wide cross between rye and wheat. Various *in vitro* techniques and/or chemical treatments may be needed to assist the recovery of fertile hybrid progeny (see Appendix 2).

Mutagenesis or mutation breeding uses either radiation or chemical mutagens to introduce mutations into the genome and thus increase genetic variation (reviewed in Çelik and Atak 2017; Kodym and Afza 2003). The DNA damage caused by mutagenesis can be repaired by natural cellular mechanisms; however, the repair is not always perfect. Multiple random changes to the genome can occur as a result of mutagenesis, including insertions or deletions (known as indels), inversions, translocations, single nucleotide changes (point mutations) and epigenetic changes. These changes are similar to those that occur naturally, although their frequency is increased. Mutation breeding has generated over 3,000 varieties

[7] Photograph by John Doebley CC BY 3.0, https://creativecommons.org/licenses/by/3.0, via Wikimedia Commons; the photograph was cropped and the background changed to white

of commercial plants (IAEA Mutant Variety Database[8]). For example, the barley variety 'Diamant' was developed via irradiation mutagenesis (IAEA Mutant Variety Database[9]). It has been used extensively since its registration in 1965, including as a parent in the development of numerous new hybrids (reviewed in Ahloowalia et al. 2004).

A number of *in vitro* techniques may be used as part of the breeding or propagation process for many food organisms such as animals (e.g. *in vitro* embryo production and cloning), plants (e.g. classic plant tissue culture techniques such as embryo rescue, chromosome doubling, somatic hybridisation) and microorganisms (routine culture techniques). The process of *in vitro* culture in and of itself can induce a variety of changes in the genome (called somaclonal variation), leading to increased genetic or epigenetic variation similar to naturally occurring changes (reviewed in Ghosh et al. 2021; Loyola-Vargas and Ochoa-Alejo 2018).

Grafting involves joining a rootstock cut from one plant to the upper part (scion) cut from another plant. It is a technique routinely used in horticulture. The characteristics of the rootstock can influence the characteristics of the scion and the associated fruit. Various macromolecules and proteins can move between the rootstock and scion, and influence plant architecture (reviewed in Thomas and Frank 2019). For example, cherry trees can be grafted onto a suitable rootstock to produce a dwarf phenotype.

2.3 Unintended changes

As discussed in Section 2.1 and Section 2.2, the improvement of food organisms relies on genetic variation and the purposeful selection of organisms with desirable characteristics (reviewed in Meyer and Purugganan 2013). The changes that occur to genomes as a result of this process are not always related to the original intent, or the desirable characteristic that has been selected for. As a result, organisms with improved characteristics may be selected that also contain other genome changes. These changes are generally referred to as unintended changes.

Appendix 3 summarises the main approaches to genetic improvement of food organisms and the types of unintended changes that can occur with each method.

As discussed in Section 2.1 and Section 2.2, the improvement of food organisms relies on genetic variation and the purposeful selection of organisms with desirable characteristics (reviewed in Meyer and Purugganan 2013). The changes that occur to genomes as a result of this process are not always related to the original intent, or the desirable characteristic that has been selected for. As a result, organisms with improved characteristics may be selected that also contain other genome changes. These changes are generally referred to as unintended changes.

Unintended changes

Unintended changes commonly occur with all methods for modifying genomes. They can be beneficial, neutral or harmful to the organism.

Whether a change is unintended or intended is unimportant for food safety. The only thing that matters is its impact on the food. For example, whether the characteristics of the organism or the food have been changed in a way that affects food safety.

Food safety is also addressed by breeders selecting for beneficial changes and discarding those resulting in potentially unsafe foods.

[8] https://mvd.iaea.org/#!Search; accessed 17 May 2021
[9] mvd.iaea.org/#!Variety/1217; accessed 17 May 2021

Unintended changes to food organisms

Unintended changes that arise through genetic improvement practices may be harmful/undesirable, neutral or beneficial with respect to an organism (Table 1). An unintended change in food organisms may add, lose or modify a particular characteristic. Beneficial unintended changes are relatively rare, and unintended changes that result in obvious harmful/undesirable characteristics will be selected against (reviewed in Arber 2010; Schnell et al. 2015).

Table 1. Unintended changes to food organisms

Unintended change	Conventional breeding	References
Harmful/ undesirable	The breeding of barley for resistance to powdery mildew has inadvertently increased the plant's susceptibility to other plant pathogens, as well as reducing its yield.	(Cellini et al. 2004; McGrann et al. 2014)
Neutral	The domestication and breeding of corn has seen a large number of genetic changes. Some of the changes introduced were not related to the original intent of the breeding, but they are of no consequence to the food organism.	(Hake and Ross-Ibarra 2015; Hufford et al. 2012)
Beneficial	Interspecific hybridisation[10] that occurred during the domestication of crops such as rice, maize and apples may have inadvertently led to the introduction of novel and superior quality traits.	(Gregory 2009; Purugganan 2019)

For example, any plant lines exhibiting undesirable phenotypic characteristics[11] such as stunting, reduced yield or reduced vigour are discarded during plant development (reviewed in Glenn et al. 2017). The majority of unintended changes to the genome however do not result in any obvious phenotypic changes to the organism and are not actively selected against. Such changes are therefore carried through to subsequent generations. These neutral changes contribute to the genetic diversity of organisms from which foods are derived.

Unintended changes to food

As with the organism itself, unintended changes may also occur to food which are harmful/undesirable, neutral or beneficial (Table 2). Organisms producing food with harmful/undesirable changes would be selected against and discarded from breeding programs by developers before commercialisation, or removed *post hoc*. Plant developers, for example, are experts in their particular plant species; they know the natural range of important analytes and which analytes to pay close attention to during breeding, e.g. key nutrients, anti-nutrients, natural toxicants and allergens. Over many years, developers will grow their plants in many different environments and continually remove any plants with harmful/undesirable nutrient changes in the food. Such screening and selection processes are a standard component of breeding programs (reviewed in Kaiser et al. 2020) and contribute to the safety of new food products (Section 2.4).

[10] Interspecific hybridisation refers to the crossing of two species from the same genus. This has occurred multiple times in the domestication of plants and animals, and usually exploits the natural genetic diversity that is present in the wild species as a result of spontaneous mutations.

[11] In plant breeding these are commonly referred to as 'off-types'.

Table 2. Unintended changes to conventional food

Unintended change	Conventional breeding	References
Harmful/ undesirable	Potatoes naturally produce glycoalkaloids (GAs), which can be toxic to humans if consumed at high levels. In one particular conventional breeding program, the levels of GA unintentionally increased to such a level that the potato had to be withdrawn from the market. Since then, acceptable levels for GAs have been defined and potatoes are routinely analysed for GA content.	(Friedman and Dao 1992; Kaiser et al. 2020; Omayio et al. 2016)
Neutral	Red peppers have been subject to selection and breeding for desirable traits such as increased yield and disease resistance for thousands of years. This has inadvertently resulted in variation in nutrient composition when these varieties are grown in different environments. While unintended, this nutrient variation is not harmful to consumers.	(Kim et al. 2019; Qin et al. 2014)
Beneficial	Vitamin A deficiency is associated with significant morbidity and mortality and is the leading cause of preventable childhood blindness. Foods high in provitamin A carotenoids, such as β-carotene, can counteract Vitamin A deficiency. β-carotene levels in bananas are highly variable, with some varieties having quite high levels. While these varieties have been cultivated for many other reasons, e.g. yield, taste and disease resistance, beneficially high levels of provitamin A carotenoids may have unintentionally been introduced or maintained.	(Englberger et al. 2003; WHO 2009)

While unintended genome changes are an expected consequence of all methods for genetic improvement, they do not necessarily alter the phenotype of the organism, or lead to changes to the characteristics of derived food products. Furthermore, an unintended change to phenotype does not automatically translate to a health or safety concern in derived food (Schnell et al. 2015). Whether a phenotypic change to an organism results in a food safety concern is dependent on the nature of the change that has occurred, not whether the change was intended or unintended.

2.4 Conventional food as a benchmark for safety

While not all food is inherently safe, conventional food has a presumption of safety which means it is considered safe on the basis of human experience (Kato-Nitta et al. 2019; Prakash 2001), i.e. there is a long history of safe human consumption.

Standard practices are in place in breeding programs that contribute to a safe food supply (Kaiser et al. 2020). In the early development stage, food organisms are thoroughly screened and selected to eliminate undesirable characteristics and ensure an elite genetic background. Undesirable food constituents that are a known hazard are routinely monitored by breeders for their production and accumulation. This ensures the levels of undesirable food constituents do not exceed acceptable limits. Backcrossing is another standard breeding practice that moves one or a few genes of interest into an adapted or elite variety while at the same time also removing many random or uncharacterised mutations (Figure 3) (reviewed in Sharma et al. 2019, 2019; Yore et al. 2018). Furthermore, breeding programs generally fortify their germplasm[12] collections with disease resistance traits to protect against

[12] Germplasm refers to living tissue that can derive entirely new plants, e.g. seeds or a few plant cells.

microbial disease causing yield loss, providing the added benefit of preventing mycotoxin contamination of foods (Kaiser et al. 2020).

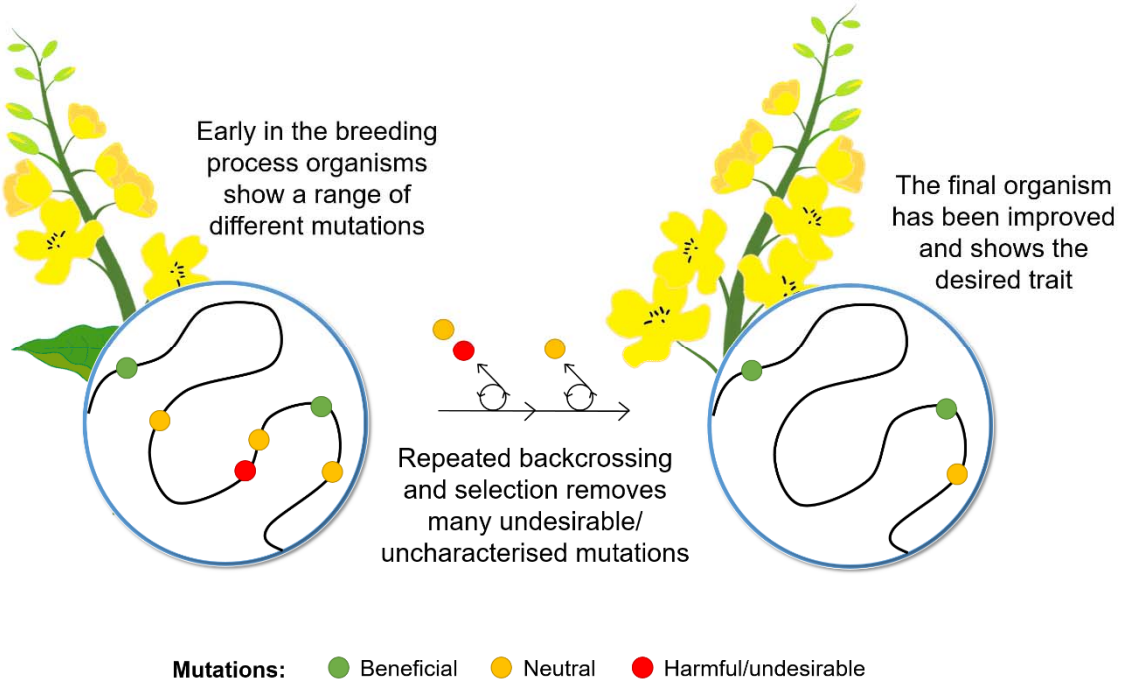


Figure 3. *Backcrossing, screening and selection to remove undesirable characteristics*

In addition to these standard breeding practices, various regulations are in place to ensure a safe food supply. These include the requirement that all food must be safe and suitable under the various Food Acts[13], as well as various food standards, such as the food safety standards[14], and standards for allergen labelling[15], microbial limits[16], maximum residue levels[17], maximum levels for contaminants[18], and hygienic food practices[19].

As discussed in previous sections, conventional methods for the genetic improvement of organisms can result in substantial changes to genomes, including a large number of unintended changes. Despite this, such methods have been used for millennia to produce safe food, i.e. the changes to the genome have in most cases been of low consequence in terms of food risk (see example in Figure 4). The screening and selection processes that are routinely applied by food developers also provide an additional measure to identify and eliminate any unwanted or undesirable changes. As a consequence, only very rare examples exist where food safety has been affected due to unintended genetic changes (Prakash 2001).

[13] https://www.foodstandards.gov.au/about/foodlawandtreaties/pages/default.aspx
[14] Standard 3.1.1: Food safety standards – interpretation and application
[15] Standard 1.2.3: Information requirements – warning statements, advisory statements and declarations
[16] Standard 1.6.1: Microbial limits in food, in combination with Schedule 27
[17] Standard 1.4.2: AgVet chemicals, in combination with Schedules 19-21
[18] Standard 1.4.1: Contaminants and natural toxicants, in combination with Schedule 19
[19] Standard 3.2.2: Food safety practices and general requirements

Red grapefruit was developed using classical mutagenesis (reviewed in Hensz R.A. 1991). This technique typically generates a much greater number of mutations than would occur spontaneously (Section 2.2 and Appendix 3). The induction of double-stranded DNA breaks is random and the resulting mutations are uncharacterised. However, food derived using classical mutagenesis methods, like red grapefruit, have a long history of safe human consumption.

Figure 4. *Classical mutagenesis in food improvement*

2.5 Conclusion

Conventional methods for food improvement exploit a variety of natural and artificial mechanisms that increase genetic variation in populations of food organisms. This genetic variation is required to achieve gains in yield, quality and other traits. Organisms with undesirable characteristics, including in derived food products, are eliminated through screening and selection, while those with improved characteristics are retained. A large number of substantial genetic changes (both natural and induced, intended and unintended) have occurred or have been introduced to organisms over time, contributing to the domestication, variety and improvement of species for human food use. Despite these significant changes to genomes, food derived from these organisms has a long history of safe human consumption. The overwhelming evidence supports the conclusion that conventional food with a history of safe use is an appropriate benchmark against which to compare other foods.

3. Food derived using new breeding techniques

NBTs is an umbrella term for a wide variety of new techniques that are being used to modify the genomes of organisms. Current examples of NBTs include: genome editing, GM rootstock grafting, cisgenesis and intragenesis. While not strictly speaking a NBT, techniques producing null segregants are also included under the NBT umbrella. As technology develops, the list of techniques considered to be NBTs is likely to expand.

This part of the assessment considers the characteristics of a broad range of NBT foods, and their similarity or otherwise to conventional food. Given its current predominance, particular emphasis has been placed on genome editing, including potential unintended changes that may arise from its use.

3.1 Equivalence to conventional food

Conventional food and GM food safety assessments

In order to examine the risk equivalence between a NBT food and a conventional food, it is helpful to first frame this assessment in the context of GM food.

Conventional food has an established history of safe use and is routinely used as the benchmark for safety in GM food safety assessments (Codex 2009; FSANZ 2019). If a GM food is comparable to a conventional food in terms of its key characteristics[20], and the introduced genetic modification has not itself created new hazards, then the GM food is considered to be "as safe as" the comparator food. This concept is widely adopted by governments around the world and is referred to the *comparative approach*.

The *comparative approach* involves comparing a GM food to a conventional counterpart (non-GM) food with a history of safe use. The aim is to identify differences, which are then further assessed to determine if the differences result in a new or altered hazard. The comparison focuses on new substances that may be present in the GM food as well as its nutrient composition. If a new or altered hazard is confirmed, further assessment is done to characterise the risk and consider appropriate risk management measures.

Extensive empirical evidence gained from twenty-five years of regulatory experience assessing GM food has demonstrated the foods assessed to date are as safe for consumers as non-GM counterpart foods. These assessments have also unequivocally confirmed the risk to consumers from unintended changes is no greater for GM food than it is for conventional food, and that in both instances the risk is low.

Applying the concept of equivalence to NBT food

The type of genome changes introduced using NBTs are the same as the types of genome changes that occur spontaneously through natural processes (Section 2.1) or induced using conventional methods (Section 2.2). These changes typically do not result in the presence of foreign DNA in the genome of the final organism used for food[21]. As a result, NBT food may be similar, or in some cases indistinguishable, from conventional food.

This leads to the hypothesis that NBT food with equivalent product characteristics to conventional food would also be equivalent in risk.

[20] Key characteristics are those of most relevance to human health or safety, including key nutrients, anti-nutrients, toxicants and allergens.

[21] Genome editing can be used to insert foreign DNA at specific sites in a genome. This would be considered a targeted form of transgenesis.

To test this hypothesis, a *prima facie* comparison of potential NBT foods to conventional food was undertaken (Appendix 4). This comparison was discussed in the final report for the *Review of Food derived using New Breeding Techniques* (FSANZ 2019). The comparison focussed on the presence of foreign DNA in the final organism used for food as well as the potential for novel characteristics to be present in derived food products. A food characteristic is considered novel if it has not previously been present in a conventional food, or if it has been altered and now falls outside the documented biological range for conventional food. Transgenesis, which is not an NBT but is used as the primary technique to produce GM food, was also included in the comparison as a point of contrast.

The analysis confirmed that a variety of different outcomes for food are possible using NBTs and in some cases these outcomes will be equivalent to those achieved using conventional methods. This is particularly the case for food derived from null segregants, as well as food derived using cisgenesis.

For other techniques such as genome editing, equivalence to conventional food will depend on whether the change to the genome results in an novel characteristic in the food. For example, modification of a known allergenic protein to alter allergenicity or production of a novel metabolite could be achieved by genome editing. At the present time, genome editing is primarily being used to introduce pre-existing traits into new varieties (e.g. the polled trait in cattle or high oleic acid trait in crops). Whether a food from genome editing is equivalent to conventional food will therefore depend on the trait that is introduced or modified. This is also the case for GM rootstock grafting and intragenesis, noting that for intragenesis foreign DNA may also be introduced.

While not part of these considerations, our analysis demonstrated how some foods derived from transgenesis may have equivalent characteristics to conventional foods. This would only apply to those foods that are refined in such a way that any novel DNA or novel protein arising from the foreign DNA insertion is no longer present in the final food.

While similarity in product characteristics can be demonstrated between NBT and conventional foods in some cases, this assessment did not explicitly consider the impact of unintended changes arising from the use of NBTs. This issue is considered further below specifically in relation to genome editing.

3.2 Unintended changes from genome editing

Overview of genome editing

Genome editing is a technique for making targeted changes to the genome of an organism, and can be accomplished using a diverse range of tools adapted from nature (reviewed in Gao 2021 and summary presented in Appendix 5). The types of changes introduced using genome editing range from single base pair changes through to the introduction, deletion or inversion of whole genes[22], regulatory elements or chromosomal regions.

The main advantage of genome editing compared to older methods (e.g. cross-breeding, classical mutagenesis and transgenesis) is the ability to target a defined modification to a specific site within the genome. The targeted nature of genome editing is often cited as an argument for its relative safety compared to other less targeted techniques. However, like other forms of genetic improvement, genome editing may also be associated with off-target and other unintended changes to a genome, which is often raised as a safety concern (e.g. Carroll 2019; Dockrill 2018; Klausner 2018; Le Page 2018).

[22] Genome editing used to insert foreign DNA is considered to be a targeted form of transgenesis.

Different types of unintended changes

As discussed in Section 2.3, the occurrence of an unintended change is a normal part of the genetic improvement process, irrespective of the approach used. This is also the case with genome editing, with most attention focussing on the occurrence and risk of a specific type of unintended change, called an off-target change. Other types of unintended changes are also possible from genome editing and these different types are discussed below.

NBTs and unintended changes

NBTs are new techniques for modifying genomes. Like other methods for modifying genomes, NBTs can result in unintended changes.

The types of unintended changes that result from NBTs are no different to the unintended changes from conventional breeding, established GM techniques, or that happen naturally.

The unintended changes arising from NBTs, including genome editing, are unlikely to pose a greater food risk compared to those arising from other methods.

(i) Off-target changes

An off-target change is a change made at a site other than the intended target site in the genome (Figure 5). This can occur when there is a degree of similarity in the DNA sequence between the target and off-target sites. Off-target changes may include point mutations, indels, large deletions, inversions and translocations. Large deletions, inversions and translocations are more likely when DNA has been cut with a nuclease, such as with the zinc finger nucleases (ZFNs), transcriptional activator-like effector nucleases (TALENs) and CRISPR-Cas tools (Appendix 5). These types of changes are not unique to genome editing, as they occur naturally (Section 2.1) and from the use of conventional methods (Section 2.2). Unintended insertions of DNA may also occur. This is a specific type of unintended change discussed in (iii) below.

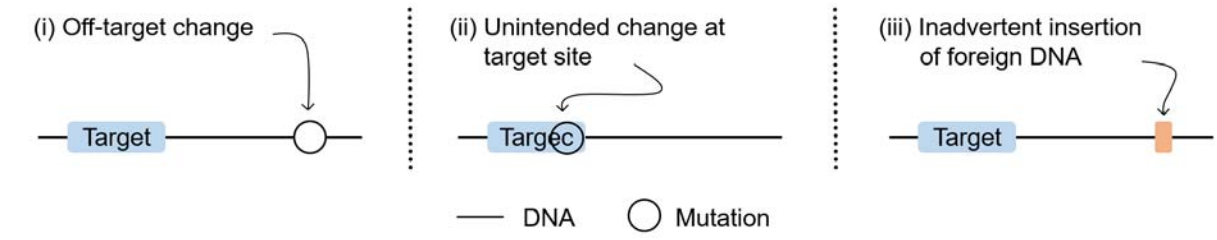


Figure 5. *Different types of unintended changes from genome editing*

(ii) Unintended changes to DNA at the target site

While the purpose of genome editing is to make an intended change to a specific target site, sometimes an additional change to the target site can occur (Figure 5). For example, when DNA has been cut with a nuclease, an unintended change may result because the DNA repair process is not perfect (Appendix 2). A mismatching of nucleotides could occur or an indel may result. These types of unintended changes are no different to unintended changes that occur through conventional methods or natural processes.

(iii) Inadvertent insertion of template or plasmid DNA either at the target site or at other sites in the genome

While many of the intended and unintended changes introduced using genome editing are identical to those that occur naturally or through conventional methods, a point of difference is the use of a template molecule (Appendix 5) or plasmid DNA in the genome editing process. Either of these foreign DNAs may become incorporated into the genome at the target site or other sites in the genome (Figure 5). This is an inadvertent form of transgenesis.

An example of this type of unintended change from genome editing was recently documented. In this case, TALENs was used to introduce the Celtic polled allele (*Pc*) in dairy cows, a trait found in hornless cattle breeds (Carlson et al. 2016). DNA sequence analysis revealed the presence of plasmid DNA, including an antibiotic resistance gene, at the target site (Norris et al. 2020a, 2020b). Insertions at off-target sites were not detected. As the introduced polled allele and the plasmid DNA were not genetically linked, the unintended change was able to be segregated away by conventional breeding, resulting in individual offspring only carrying the polled allele.

Managing the incidence of unintended changes

Unintended changes occur regardless of the method used to genetically improve an organism. Through experimentation, breeders, developers and scientists have established strategies to minimise the occurrence or impact of these changes. A summary of these strategies is described below.

(i) Backcrossing

In conventional breeding, the occurrence of unintended changes can be managed through repeated backcrossing. The backcrossing process is also routinely used for organisms developed using established GM techniques or NBTs and can thus be used to reduce or eliminate off-target and other unintended changes (reviewed in Bishop and van Eenennaam 2020; Bortesi and Fischer 2015; Graham et al. 2020; Schwartz et al. 2020; van Eck 2020; Wei et al. 2015; Zhao and Wolt 2017).

(ii) Screening

Screening methods comprise both phenotypic and genotypic approaches. At the phenotypic level, screening will involve the identification of adverse traits such as reduced yield or increased levels of endogenous toxicants (e.g. glycoalkaloids). Only organisms with the most improved characteristics will be advanced. This selection process following screening is standard practice for all forms of genetically improved organisms.

Of most relevance to genome editing are the genotypic or molecular biology tools that enable the identification of organisms with unintended changes to the genome, including off-target changes (reviewed in Hennig et al. 2020; Modrzejewski et al. 2020; Wei et al. 2015). Commonly used molecular approaches include Southern blotting, targeted amplification by PCR and DNA sequence analyses. More recently developed approaches include whole genome sequencing and updated methods for Southern blotting such as Southern-by-sequencing (Zastrow-Hayes et al. 2015). This area is highly dynamic, with new or improved methods continuously being developed.

Screening by molecular approaches is greatly facilitated with the use of bioinformatics (Grohmann et al. 2019). Bioinformatics requires access to reference and pangenomic[23] sequence data. Access to pangenome sequence data is crucial for differentiating off-target changes from normal genomic variation (reviewed in Graham et al. 2020; Hennig et al. 2020). Currently, both reference and pangenomes are incomplete for many livestock, crops and microorganisms (reviewed in Sherman and Salzberg 2020) however the information gap is being narrowed through major ongoing international projects such as the 10,000 plant genome and the Genome 10K Community of Scientists projects for vertebrates (reviewed in Richard 2020).

While screening can successfully identify genomic changes, it is important to note screening cannot pinpoint whether a change was introduced through genome editing, a conventional approach or occurred spontaneously. This can be important when genome editing is combined with conventional approaches. For example, a genome edited plant tissue may be regenerated using plant cell culture techniques, known to produce somaclonal variants (reviewed in Kaeppler et al. 2000; Miguel and Marum 2011; Neelakandan and Wang 2012).

This was observed in the recent development of a herbicide-tolerant canola line. An oligo-directed mutagenesis genome editing tool was applied to canola. After selection by exposure to the herbicide and regeneration by tissue culture, characterisation of the trait indicated it was more likely to have resulted from somaclonal variation, rather than oligo-directed mutagenesis (Health Canada 2016).

(iii) Design

Optimisation of the editing tools and methodology also may reduce the incidence of unintended changes. Bioinformatics permits identification of putative off-target sites and the probability of their occurrence can be calculated (reviewed in Bao et al. 2021; Sledzinski et al. 2020; Tycko et al. 2016). Through identification, the correct tools can be chosen, the components such as guide templates and nucleases can be redesigned prior to use and the putative sites specifically characterised post-editing. Further technological developments in genome editing, such as the identification of novel nucleases and the reengineering of existing nucleases may also contribute to a reduction in the incidence of unintended changes (Shivram et al. 2021; Tóth et al. 2020).

3.3 Conclusion

A large variety of genome changes can be generated using NBTs. Many of these changes will be identical to those introduced using conventional breeding or that occur spontaneously. NBTs can also be used to introduce genome changes that are identical to those introduced using established GM techniques.

In terms of unintended changes, those arising from NBTs are no different to the unintended changes that may arise through conventional breeding, GM techniques, or changes that occur spontaneously. Such changes are a normal outcome of genetic modification, no matter what method is used, and have not been identified as a significant source of risk for either conventional food or GM food. Furthermore, approaches are routinely used to screen for and select against unintended changes, as well as minimise their occurrence, which further reduces any potential risk. The unintended changes arising from NBTs, including genome editing, are therefore unlikely to pose a greater food risk compared to those arising from other methods.

[23] a pangenome combines all known reference genomes to allow comparison across the range of genetic diversity within a species

Since the types of genome changes (both intended and unintended) introduced using NBTs can be identical to those introduced using conventional methods, it follows that some NBT food will be similar, and in many cases identical, to conventional food. When an NBT food is equivalent in its product characteristics to conventional food with a history of safe use, the NBT food can be considered to also be equivalent in risk, or "as safe as" conventional food.

4. Discussion

In order to revise the definitions for 'food produced using gene technology' and 'gene technology' under this proposal, it must first be decided whether each new NBT food should be subject to pre-market assessment and approval. This approach is applied to GM food and was based on the presumption that GM food may pose a greater risk compared to conventional food.

The question about the need for pre-market assessment of NBT food is therefore essentially a question about risk, and how NBT food compares to conventional food. If it can be demonstrated that NBT food is equivalent in risk to conventional food, then it may be argued that a pre-market safety assessment is unnecessary.

This assessment therefore set out to determine how NBT food compares to conventional food. The assessment confirmed that over time, many considerable genetic changes have been introduced to food organisms using conventional breeding. Despite this, conventional food has a long history of safe human consumption and as a consequence is typically not subject to pre-market assessment and approval. In other words, genetic modification *per se* has not been observed to be a significant source of risk for conventional food. Furthermore, the screening and selection processes used in breeding, in combination with existing food regulations, appear sufficient to manage any risks that may arise through conventional breeding.

When investigating genetic changes caused by NBTs, or more specifically genome editing, the assessment established that changes introduced using NBTs are directly comparable to those introduced through conventional breeding or that happen naturally. Furthermore, the unintended changes to the genome are identical to those from conventional breeding, or that occur using GM techniques. No evidence for novel or unique types of genetic changes, either intended or unintended, have been found. This is not surprising given that DNA is a well-characterised substance which can only change in a given manner.

These findings illustrate that when assessing the risk from NBT food, the size of a genetic change, whether it was intended or not, or the method used to effect the genetic change are irrelevant considerations. The crucial factor from a food safety perspective when any genetic change is made is the *impact* of that change on the food. If a genetic change is made using an NBT, and the introduced change has not resulted in new or altered product characteristics compared to conventional food, it can be concluded the NBT food will carry the same risk as the equivalent conventional food. Similarly, refined ingredients derived from GM food which have no novel DNA, novel protein or altered product characteristics will also be identical in risk to the conventionally produced counterpart. This provides a clear basis for excluding these foods from a requirement for pre-market safety assessment as a GM food.

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Appendix 1 Natural mechanisms leading to genetic variation in organisms

DNA replication and repair

Maintaining the integrity of DNA in living organisms is essential for the accurate propagation of genetic information from one generation to the next. This is achieved by mechanisms for the accurate replication of DNA as well as the faithful repair of any damaged DNA.

DNA replication is a process that occurs in all organisms and involves a complex system of enzymes (DNA polymerases, helicases, ligases). The replication process is semi-conservative, meaning the DNA helix is unwound into two single strands, with each strand serving as a template for the synthesis of a new complementary strand. After synthesis of the new strands, cellular proofreading and error checking mechanisms ensure any replication errors are repaired with high accuracy.

Most organisms have multiple DNA polymerases. The main DNA polymerase in *Escherichia coli*, DNA polymerase III, has very high fidelity arising from its ability to efficiently select correct nucleotides for the polymerisation reaction and to remove any incorrect nucleotides. For example, DNA replication in *E. coli* chromosomal DNA averages only one error for every 10^9 –10^{10} copied bases (Arana and Kunkel 2010; Schaaper 1993). Other DNA polymerases, however, can have lower fidelity, often from reduced proofreading ability (Arana and Kunkel 2010).

While mutations due to DNA replication are rare, they can be passed on to offspring, and their accumulation over time can lead to significant genetic variation in populations. For example, it has been reported that single nucleotide polymorphisms (SNPs)[24] are detected every 48-2,000 base pairs in wheat, soybean and maize (Weber et al. 2012). In fish, a spontaneous mutation rate of less than $1x10^{-6}$ per specific locus has been reported (Kuroyanagi et al. 2013).

DNA is exposed to many factors that can cause damage (Cadet and Wagner 2013). This includes external factors, such as environmental, biological, physical and chemical agents[25] (Murray and Carr 2018). DNA is also exposed to hydrolysis in the aqueous cellular environment, and to oxidation caused by metabolites of normal cellular processes, such as respiration or photosynthesis[26] (reviewed in Chatterjee and Walker 2017; Murray and Carr 2018).

DNA has been estimated to incur damage tens of thousands of time per cell per day by these various external and internal agents (reviewed in Gao et al. 2017; Giglia-Mari et al. 2011). The types of damage includes formation of apurinic/apyrimidinic (AP) sites, interstrand crosslinking, single- or double-stranded DNA breaks, DNA-protein crosslinking and base substitutions. If left unrepaired, formation of AP site base substitutions may cause small changes (point mutations or small deletions) to the DNA, and may also lead to genomic instability. However, double-stranded DNA breaks are of most consequence if unrepaired as they disrupt DNA replication and other fundamental cell cycle processes.

To maintain the function and integrity of DNA, all organisms have evolved several DNA proofreading and repair mechanisms (Spampinato 2017). Each mechanism relies on the

[24] Refers to DNA sequence variation between individuals in a population or species that occurs at a single nucleotide position in a genome.
[25] For example, ionising and non-ionising radiation, various chemicals, physical shearing, cellular metabolites or viruses.
[26] For example, reactive oxygen species.

detection of an error or fault in the DNA; assembly of various factors, co-factors and enzymes; patching; and nick sealing. While these mechanisms are generally of high fidelity, errors in repair (if they are not lethal) may also contribute to genetic diversity in populations.

Polyploidy

Polyploid organisms usually arise as a result of a rare mitotic or meiotic event, such as nondisjunction[27], which causes the formation of gametes that have a complete set of duplicate chromosomes (reviewed in Leitch and Leitch 2008). In addition to these natural mechanisms, polyploidy can be artificially induced in plants and fungal cell cultures via treatment with mitotic inhibitors such as colchicine (reviewed in Touchell et al. 2020). Artificial whole genome duplication was introduced as a conventional breeding method in the 1930s.

Whole genome duplication is associated with a wide range of changes such as gene loss and gene silencing, epigenetic changes such as changes to DNA methylation, changes in transposon activity, as well as cellular-level modifications and chromosomal-level changes (reviewed in Soltis and Soltis 2021). Examples of polyploid plants include potatoes, wheat, oats and strawberries (reviewed in Kyriakidou et al. 2018). Examples of stable polyploid food animals include salmon and carp (reviewed in Le Comber and Smith 2004).

Mobile genetic elements

Different types of mobile genetic elements exist among both eukaryotic and prokaryotic organisms. These include transposons, plasmids, and bacteriophages and other viruses.

Transposons are recognised as one of the major contributors to the evolution of all prokaryotic and eukaryotic organisms. They were originally discovered in plants by Barbara McClintock in the mid-1900s (McClintock 1950) but have since also been found in animals and microorganisms. Transposons can move in the genome either by a copy-and-paste mechanism (class I transposons, also called retrotransposons) or a cut-and-paste mechanism (class II transposons). Some transposon sequences have originated outside the organisms they are found in, e.g. certain class I transposon sequences are related to retroviral sequences (reviewed in Wells and Feschotte 2020). Class I transposons are especially prone to generating highly repetitive sequences. Some class II transposons move into and out of specific loci.

As transposable elements may insert anywhere in the genome, insertional effects might be observed such as changes to genome architecture and size; chromosomal rearrangements, alteration of gene expression; and mobilisation of endogenous sequences (reviewed in Fambrini et al. 2020). While transposons have played an essential role in the evolution of organisms, most, but not all, transposons are either dormant or inactive. This is mainly the result of epigenetic silencing mechanisms as well as transposon autoregulatory measures that suppress transposon activity, which, if unchecked, may be deleterious to an organism.

Plasmids are small extrachromosomal DNA elements that are mostly present in prokaryotes, such as bacteria (Brooks et al. 2019). Plasmids are one of the key agents leading to bacterial genetic diversity, genome evolution and the acquisition of new information for fast adaptation to new environments or stressful conditions. They can be passed down to daughter cells during cell division (reviewed in Ebersbach and Gerdes 2005) or transferred to different bacterial species (horizontal DNA transfer) via conjugation[28]. Plasmids can enable bacteria to live in hostile environments, such as those with high levels of heavy metals or antibiotics, e.g. through the acquisition of antimicrobial resistance genes. Plasmids also contribute to

[27] The failure of homologous chromosomes or sister chromatids to separate properly during cell division.
[28] The transfer of genetic material between bacterial cells by direct cell-to-cell contact or by a bridge-like connection between two cells.

virulence, causing disease in bacterial hosts and, thus, affecting evolution of their avirulence genes.

Bacteriophages infect their bacterial host and may have either detrimental or temperate effects. Bacteriophages can persist as prophages in the genomes of bacteria, and have been reported to constitute up to 20% of the genome (reviewed in Bordenstein and Reznikoff 2005; Canchaya et al. 2003). Prophage sequences may contribute to sequence diversity between bacterial isolates (reviewed in Bossi et al. 2003). Upon activation, prophages excise themselves from their host's genome and can lead to cell lysis. Lysis resistant bacteria will be able to multiply. Inaccurate excision will either leave sequence remnants or introduce deletions in the bacterial DNA. Thus, bacteriophages can affect both bacterial population dynamics and genome evolution.

Changes to the genome from mobile genetic elements, both within chromosomes as well as in the form of extrachromosomal DNA is common in a wide range of food organisms, e.g. lactobacilli (Wang and Lee 1997), corn (McClintock 1950), pigs and cattle (Rodriguez-Terrones and Torres-Padilla 2018), and mushrooms (Castanera et al. 2017).

Sexual reproduction

Genetic variation arises through the process of meiosis, specifically the independent assortment and crossing over of chromosomes in the germ cells of sexually reproducing organisms, as well as the random union of gametes during fertilisation.

The first phase of meiosis begins with a diploid parent cell that undergoes one round of DNA replication followed by two cycles of nuclear division. This results in four haploid cells, the gametes. The production of gametes is usually necessary before fertilisation can occur in order to maintain the correct ploidy level in the offspring (reviewed in Ziolkowski and Henderson 2017; for exceptions see section on ploidy). During this process, homologous chromosomes physically pair and recombine their genetic material by crossing over. At the start of a crossover, double-stranded DNA breaks are deliberately induced in all four DNA strands. The double-strand breaks are repaired and result in a crossover in two strands of the homologous chromosomes, while the other two DNA strands remain unaffected (Lambing et al. 2017). Crossovers can result in the transfer of a large number of genes onto the homologous chromosome. Exposure of organisms to stress has been shown to increase the rate of crossovers (Si et al. 2015).

Appendix 2 Additional information on conventional breeding

Simple selection and domestication

During early domestication, plants and animals would have resembled their wild counterparts. Over time, as continued selection resulted in significant changes in important characteristics (e.g. non-shattering seed and increased seed size in cereals), the differences between domesticated and wild varieties became greater. Archaeological evidence currently suggests a 2,000-2,500 year timeframe to achieve those fundamental initial changes in important cereals (reviewed in Purugganan 2019).

In addition to simple selection, increasing evidence supports that hybridisation of partially domesticated species with wild species, leading to polyploidisation, played an important role in the diversification of plant species, evolution of genes and the domestication of crops (Zhang et al. 2019). An example of hybridisation of a partially domesticated species with a local wild species is *Indica* rice (reviewed in Purugganan 2019). Similarly, introgression of DNA sequences from early domesticated pigs from Eastern Anatolia into local wild boars in Western Anatolia can be detected in ancient DNA (reviewed in MacHugh et al. 2017).

While some traits evolved in parallel across different environments during the domestication of crops, such as seed retention, plant stature, reduced bitterness of fruit and increased seed size, other traits were consistently diversified, e.g. fruit colour. Similarly, common traits in domesticated mammals include coat colour variation, docility, and a reduction in brain size (reviewed in MacHugh et al. 2017).

Cross-breeding in plants and animals

The deliberate crossing of sexually compatible plants or animals in order to combine superior traits which are expressed in the offspring is a development that followed domestication.

Chromosome reassortment and other sources of genetic variation occur in cross-breeding. For example, a number of chromosome rearrangements, such as inversions or deletions have been shown in maize using chromosome-scale genome assemblies, and amongst other large changes, an approximately 75.5Mb inversion was found in chromosome 2 in three of 66 pangenome assemblies generated from inbred lines (Schwartz et al. 2020).

Wide crosses can be made between more distantly related plants or animals that do not normally sexually reproduce with each other and in some cases specific *in vitro* techniques (see below) may be required to recover progeny. Wide crossing is usually done to transfer useful genes, such as disease resistance, from wild relatives, or to create entirely new varieties.

Linebreeding (in plants) and pure breeding (in animals) can narrow the genetic basis to some degree leading to an accumulation of deleterious alleles in a population causing inbreeding depression (reviewed in Howard et al. 2017; Mackay et al. 2021). However, cross-breeding two inbred lines typically results in offspring with improved function compared to the parents. In plants, this disproportionate increase in improvement is known as the heterosis effect and is the reason hybrids are widely used in commercial seed production.

Mutagenesis

Mutagenesis is an important method that has been widely used on numerous food organisms since the 1940s. This practice is often referred to as mutation breeding. Mutation breeding

uses either radiation or chemical mutagens to introduce mutations into the DNA and hence increase genetic variation (reviewed in Kodym and Afza 2003). Ionising radiation is by far the most common method used (reviewed in Ahloowalia et al. 2004; Çelik and Atak 2017). Mutagenesis is commonly used to improve microorganisms used in food production, including various fungi and bacteria (e.g. *Oenococcus oeni* (Li et al. 2015) or *Aspergillus niger* (Lotfy et al. 2007b)).

The type of changes to the genome are identical to those that may occur naturally, although their frequency is increased. For example, the mutation rate in offspring of X-ray irradiated male mice can increase by over 20-fold compared to the non-treated control group (reviewed in Probst and Justice 2010). Similarly, the chemical mutagen N-ethyl-N-nitrosourea can cause (mainly point) mutations at a rate two hundred times higher than occur spontaneously (reviewed in Probst and Justice 2010). Mutagenised organisms undergo repeated rounds of propagation and selection for improved characteristics. In plants, a number of rounds of backcrossing to one parent ensures that off-type plants, such as those with stunted growth or low yield, are removed.

In vitro techniques

A number of *in vitro* techniques may be used as part of the breeding or propagation process for many food organisms. While many microorganisms are routinely maintained *in vitro* during food production, there are also a number of important *in vitro* techniques used in both plant and animal breeding, such as *in vitro* fertilisation and embryogenesis; embryo rescue; somatic hybridisation, cybridisation and animal cloning.

Microorganisms have been cultivated *in vitro* for millennia, e.g. during fermentation, including the production of alcoholic beverages facilitated by yeasts (e.g. *Saccharomyces cerevisiae*) and molds (e.g. *Botrytis cinneraea*); dairy products, including yoghurt (e.g. *Lactobacillus bulgaricus*); and soy sauce (e.g. *Aspergillus oryzae* or *Asp. sojae*). More recently, a wider variety of organisms have been selected for use, e.g. in the production of enzymes or organic acids (Lotfy et al. 2007a and b; Muensean and Kim 2015).

In cattle and sheep, ovum pick up, *in vitro* oocyte maturation, *in vitro* fertilisation and *in vitro* embryo culture[29] is followed by implantation into a recipient female, pregnancy and delivery. While only part of the life cycle occurs *in vitro*, this process shortens the generation interval and increases the genetic presence of the maternal side by enabling the female to produce more progeny per year (reviewed in Boni 2012; Ward et al. 2000). *In vitro* embryo production is in routine use in animal husbandry and commonly contributes to food production. Genetic changes are similar to cross-breeding.

Specifically in plants, a cross between a diploid and a tetraploid, between two different species (interspecific) or two different genera (intergeneric), can lead to abnormal development of the embryo *in vivo* (reviewed in Bridgen 1994). If it is possible to physically excise the immature embryo, culturing using specific *in vitro* conditions may enable a mature hybrid to form. The embryo rescue technique is an important tool that reduces the generation intervals and has produced many improved crop varieties. Haploids can be generated which can undergo whole genome duplication, either spontaneously or induced. The resulting double haploids can be used in further breeding improvements. Changes to the genome are similar to those from wide crosses.

Plant cells are totipotent, even when already fully differentiated (reviewed in Neelakandan and Wang 2012). Plant cells can dedifferentiate, proliferate and regenerate into fully mature

[29] *In vitro* embryo production is a process involving *in vitro* oocyte maturation, *in vitro* fertilisation and *in vitro* embryo culture.

plants *in vitro*, if the appropriate nutrients and hormones are supplied. This is the basis of commercial micropropagation of plants, which seeks to obtain homogeneous plant clones. Some animal cells, such as the cells of an early cell stage embryo are also totipotent and may be used in animal cloning (reviewed in Edwards et al. 2003).

Somatic hybridisation in plants, animals and fungi refers to a number of *in vitro* techniques based on the fusion of cells (reviewed in Eeckhaut et al. 2013). Somatic hybridisation occurs naturally in most fungal phyla (reviewed in Schardl and Craven 2003), and was developed for use in mammalian genetic studies, as those cells are naturally without a cell wall. In plants, the potential for their use in developing improved commercial crops, such as potatoes, *Brassia* spp. and *Citrus* spp., was only realised relatively recently (reviewed in Davey et al. 2005; Eeckhaut et al. 2013; Germanà 2006). Somatic hybridisation in plants can circumvent sexual hybridisation, produce homokaryons or heterokaryons[30], polyploids, or cells with cytoplasm from both parents. Protoplasts can also be used to establish alloplasmic hybrids, i.e. where the nucleus from one cell is introduced into a different, enucleated cell (reviewed in Eeckhaut et al. 2013). In animals, somatic cell nuclear transfer into an enucleated oocyte can be used to re-establish totipotency and, thus, be used in animal cloning. This method has low efficiency, most likely due to epigenetic effects in the resulting organism (Smith et al. 2010).

The process of *in vitro* culture can induce a variety of changes in the genome, resulting in changes similar to naturally occurring changes (Ghosh et al. 2021; reviewed in Loyola-Vargas and Ochoa-Alejo 2018). In plants, this is referred to as somaclonal variation.

Grafting

While grafting is usually considered to be a very old plant propagation technique, both shoot and root grafting can also occur naturally (reviewed in Mudge et al. 2009). For example, neighbouring trees can graft naturally once making physical contact with each other and exposing the vascular cambium. This type is termed approach grafting. The roots of trees in close proximity can also form grafts, share water and nutrients, and provide stability against high winds. However, grafts permit transmission of some pathogens.

Most types of grafting used in horticultural practice involve joining a rootstock cut from one plant, to the upper part (scion) cut from another plant. The cut surfaces are held in place close to one another until a callus has formed connecting the two parts and permitting the vascular tissue to reform, allowing the transport of water, nutrients and other solubles. This graft junction is comprised of undifferentiated cambium cells which can differentiate into xylem and phloem cells which make up mature vascular tissue. Grafts can be successful in plants of the same species or genus.

Grafting can improve fruit quality under various growing conditions as it depends on the characteristics of the root system. In some grafted plants, fruit quality characteristics, such as sugar, texture, size, and flavour can be affected by the root system. The root systems can also influence plant architecture and stature, abiotic and biotic stress tolerance and yield.

Grafting is routinely used in horticulture, particularly on fruit trees, shrubs or vines (reviewed in Melnyk and Meyerowitz 2015). Various macromolecules such as select RNAs and proteins can move between scion and root stock, and cause epigenetic effects (reviewed in Thomas and Frank 2019). Plasmodesmata enable plastid genomes and other DNA to travel a short distance beyond the graft junction (reviewed in Wang et al. 2017).

[30] Cells with more than one nucleus, where the nuclei are either genetically different (heterokaryon) or the same (homokaryon).

Appendix 3 Approaches to genetic modification

Method / Technology	Since	About	Genetic outcome	Unintended changes	References
Domestication	~10,000 BCE	Selection of seeds or animals based on human preference, agricultural practices and the environment. Relies on spontaneous/natural mutations[31].	Tens of thousands of new genes are recombined[32] and often large amounts of accompanying DNA is transferred (linkage drag).	Linked, undesired characteristics are often introduced. Pleiotropic effects[33]. Genetic diversity, fitness and nutrition affected.	(Bourque et al. 2018; Larson and Burger 2013; Purugganan 2019; Seah et al. 2007; Smýkal et al. 2018; Vitte et al. 2014)
Grafting	1,000 BCE	Artificial joining of two different plants.	Chimeric organism. Polyploid cells restricted to the area around the connecting tissue. Select RNAs and proteins affect the entire chimera.	Unpredictable changes in gene expression and phenotype.	(Mudge et al. 2009; Thomas and Frank 2019; Wang et al. 2017)
Cross-breeding	1800s	Purposeful crossing of closely or distantly related individuals based on desirable characteristics.	Similar to domestication. Wide crosses have the potential to introduce a large number of new genes and other linked DNA.	Similar to domestication. E.g. as tomato breeders selected for shelf-life, disease resistance and size the resulting tomatoes often have poorer flavour.	(Gregory 2009; Wang and Seymour 2017)

[31] For example, additional DNA, deletions and/or rearrangements occur with DNA repair or the movement of transposable elements (insertional effects).
[32] Known as meiotic recombination.
[33] When a single mutation or gene affects more than one phenotypic characteristic.

Method / Technology	Since	About	Genetic outcome	Unintended changes	References
Interspecific hybridisation requiring embryo rescue (*In vitro* culture)	1920s	The rescue of embryos that would otherwise degenerate (due to lack of endosperm) in some interspecific and intergeneric hybridisations through *in vitro* culture.	Significant genotypic variation. Can be used to produce haploids through chromosome elimination.	Similar to domestication. Somaclonal variation[34]. Haploid plants are generally less fit than their parents, e.g. reduced organ size and infertility.	(Bednarek and Orlowska 2020; Bridgen 1994; Clarke et al. 2006)
Chromosome doubling in plants (*In vitro* culture)	1930s	The use of antimitotic agents (e.g. colchicine) to induce polyploidy[35] or it can be used to make a double haploid plant[36].	Involves chromosome doubling or whole genome duplication	Somaclonal variation. Significant and unpredictable changes in gene expression and phenotype.	(Chaikam et al. 2019; Gilles et al. 2017; Touchell et al. 2020)
Classical mutagenesis	1940s	Chemicals or radiation are used to induce random mutations at a faster rate than would occur naturally, with the aim to generate desirable characteristics.	Chemical mutagens predominantly cause single base substitutions. Radiation can cause double-stranded DNA breaks and results in mixture of deletions, rearrangements and single base substitutions.	Beneficial characteristics are screened for, but the selected organism may carry additional mutations. Somaclonal variation when *in vitro* culture used, e.g. epigenetic changes.	(Holme et al. 2019; Kodym and Afza 2003; Spencer-Lopes et al. 2018)

[34] As a result of *in vitro* culture, plant cells or tissue can exhibit single base substitutions, deletions, insertions, rearrangements, chromosome number changes or activation of transposable elements.
[35] More than two sets of chromosomes.
[36] Double haploid plants restore fertility and allow developers to maintain a homozygous inbred line.

Method / Technology	Since	About	Genetic outcome	Unintended changes	References
Somatic hybridisation in plants (*In vitro* culture)	1970s	The fusion of protoplasts to produce a hybrid plant with a mixture of parental characteristics and, in some cases, to overcome reproductive barriers in plant species.	Hybrid often has chromosomes of both parents and/or the elimination of all or parts of chromosomes. New nuclear and cytoplasmic genome combinations.	Wide ranging unpredictable genetic, epigenetic and phenotypic changes. Somaclonal variation.	(Guo et al. 2010; Jia et al. 2017; Rose et al. 1990)
In vitro embryo production in animals	1980s[37]	The processes around and including *in vitro* fertilisation resulting in an embryo which can be implanted into a female animal.	Similar to domestication and cross-breeding.	Similar to domestication and cross-breeding. Somaclonal variation.	(Boni 2012; Sjunnesson 2020; Ward et al. 2000)
Transgenesis	1980s	DNA from an unrelated organism is inserted in any configuration.	One or a few new genes found in final organism.	Similar to conventional breeding.	(Ladics et al. 2015; Schnell et al. 2015)
Cisgenesis	2000s	DNA from the same or a cross-compatible species is inserted without altering its sequence or configuration.	Similar to domestication and cross-breeding.	Similar to conventional breeding.	(Espinoza et al. 2013; Holme et al. 2013; Schouten et al. 2006)
Intragenesis	2000s	DNA from the same or cross-compatible species is rearranged before being inserted.	Similar to domestication and cross-breeding, but less so than cisgenesis.	Similar to conventional breeding.	(Espinoza et al. 2013; Holme et al. 2013)

[37] *In vitro* fertilisation was successful from the 1960s; however, reliability needed to be improved.

Method / Technology	Since	About	Genetic outcome	Unintended changes	References
Genome editing	2010s	A technique which can be used to make specific changes at targeted locations in the genome of an organism.	Small or large amounts of DNA may be inserted, deleted, modified or replaced. Inserted DNA may be native or foreign.	Similar to conventional breeding.	(Graham et al. 2020; Lema 2021; McFarlane et al. 2019; Zhao and Wolt 2017)

Appendix 4 Equivalence between NBT foods and conventional foods

New breeding technique	Product characteristics		Equivalence to conventional food
	Presence of foreign DNA in the final food organism	Novel characteristic in the food[38]	
Genome editing Can be used to introduce a wide range of genome changes: 1. Some applications introduce no foreign DNA, and changes are typically point mutations, small indels, or large deletions. These types of genome changes often occur in nature. 2. Some applications are used to introduce foreign DNA. Some applications may lead to the inadvertent incorporation of template or plasmid DNA[39]. These types of changes are unlikely to occur in nature.	**Depends**	**Depends**	**Depends** Needs to be determined case-by-case. Food that does not have any novel characteristics, and where no foreign DNA has been introduced, will be equivalent to conventional food.

[38] A food characteristic is considered novel if it has not previously been present in a conventional food, or if it has been altered and now falls outside the documented biological range for conventional food.
[39] Refer to the table entry on transgenesis.

New breeding technique	Product characteristics		Equivalence to conventional food
	Presence of foreign DNA in the final food organism	Novel characteristic in the food[38]	
GM rootstock grafting A non-GM scion (upper part of a plant) is grafted onto a GM rootstock. The GM rootstock could contain foreign DNA inserted from any species. This DNA will be restricted to the rootstock and tissue around the graft. Food, such as fruit, is typically derived from the scion. Some genetic modifications to the rootstock may influence the characteristics of the scion, and potentially also the food.	**Depends**	**Depends** on whether the modification to the rootstock influences the characteristics of the food.	**Depends** Needs to be determined case-by-case. The food will be equivalent to conventional food, if no novel characteristics are introduced.
Cisgenesis DNA from the same or cross-compatible species is inserted without altering its sequence or configuration. Used to transfer traits from one variety/breed to another. Includes duplicating an existing gene or replacing/converting an allele (where one gene variant is converted to another gene variant). Cisgenesis mimics cross-breeding.	**No**	**No**	**Yes**
Intragenesis DNA from the same or a cross-compatible species is rearranged before being inserted into the genome. Genome rearrangements occur in nature. Regulatory elements from non-related species may also be included in these types of gene constructs. Mixing of genetic elements from non-related species is unlikely to occur in nature.	**Depends**	**Depends**	**Depends** Needs to be determined case-by-case. Food that does not have any novel characteristics, and where no foreign DNA has been introduced, will be equivalent to conventional food.

New breeding technique	Product characteristics		Equivalence to conventional food
	Presence of foreign DNA in the final food organism	Novel characteristic in the food[38]	
Techniques producing null segregants DNA is inserted into an organism, for example, to facilitate breeding. Towards the end of the breeding process, progeny are selected that have not inherited the introduced DNA (it does not serve any purpose in the final organism used for food). These progeny are referred to as null segregants	**No**	**No**	**Yes**
Gene Technology			
Transgenesis DNA from an unrelated organism is inserted into the genome in any configuration. Some NBTs may involve foreign DNA (e.g. genome editing, intragenesis, GM rootstock grafting). Transgenesis in the form of transposon insertion can occur in nature. In addition, natural infection of plants with *Agrobacterium tumefaciens* can result in foreign DNA insertion in rare instances.	**Yes**	**Depends**	**No** However some refined ingredients which do not contain any DNA or protein resulting from foreign DNA in the final food organism, and which do not have novel characteristics, will be equivalent to similar products from conventional food.

Appendix 5 Current tools used in genome editing

Tool	Since	About	Genetic outcome	References
Oligo-directed mutagenesis (ODM)	1980s	Targeting is achieved with a template oligonucleotide.	Insertions, deletions and point mutations.	(Beetham et al. 1999; Doetschman et al. 1987; Sauer et al. 2016; Zhu et al. 1999)
Zinc finger nucleases (ZFNs)	1990s	Targeting is achieved by sequence recognition by the zinc-finger proteins, with or without a template.	Insertions, deletions and point mutations.	(Bitinaite et al. 1998; Choo et al. 1994; Kim et al. 1996)
Meganucleases	2000s	Targeting is achieved by sequence recognition of the endonuclease.	Insertions, deletions and point mutations.	(Chevalier and Stoddard 2001; Seligman et al. 2002)
Transcriptional activator-like effector nucleases (TALENs)	2000s	Targeting is achieved by sequence recognition by effector proteins, with or without a template.	Insertions, deletions and point mutations.	(Boch et al. 2009; Moscou and Bogdanove 2009)
Clustered regularly interspaced short palindromic repeats with CRISPR-associated nuclease proteins (CRISPR-Cas)	2013	Targeting is achieved by sequence recognition by effector proteins, with or without a template.	Insertions, deletions and point mutations.	(Gao et al. 2017; Hwang et al. 2013; Jiang et al. 2013; Jinek et al. 2013)
Base editing	2016	Targeting is achieved with a template.	Point mutations.	(Komor et al. 2016; Nishida et al. 2016)
Prime editing	2019	Targeting is achieved with a template.	Insertions, deletions and point mutations.	(Anzalone et al. 2019)